Abstract

Title of Document: BEHAVIORAL AND NEUROENDOCRINE CORRELATES OF SEX CHANGE IN THE GILTHEAD SEABREAM, *SPARUS AURATA*

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Sequential hermaphroditism is the most radical form of environmental sex determination observed in fish: functional adult males or females retain their ability to change sex even as adults. Among the factors that affect sex change in these species, the least understood is the social environment. Here, I studied the influences of social context on sex change in the Gilthead Seabream, *Sparus aurata*, by using the individual's dominance rank as an indicator of social status. To understand the role that the brain might play in sex change, I also studied the two main neuroendocrine factors that serve as the sexually differentiated axes of neural plasticity in most teleost species: AVT and GnRH. To do this, I first developed a set of tools designed to address the challenges associated with observing the behavior of aquacultured species. Using these tools, I provide the first in-depth study of seabream captive behavior, including the results of size-matched and sex-matched paired encounters. I found that females are more aggressive than males, but this difference is influenced by gonadal developmental status. I also showed that small but young males are more aggressive than bigger but older females. I cloned the AVT mRNA in seabream, and validated a quantitative assay to measure total brain AVT levels together with GnRH-1, GnRH-2, and GnRH-3 levels. I found that AVT and GnRH-3

levels rise during the onset of the hypothesized sex-change window, and drop to pre-quiescent levels until spawning, when all of these factors seem to increase their expression levels again. I also show for the first time, that GnRH-2 and dominance rank are strongly correlated in seabream during the spawning season but not during quiescence. GnRH-1 was strongly correlated to rank during quiescence but not during spawning. Finally, neither dominance rank nor size were a good predictor of the outcome of sex change, which seems to contradict what has been documented in sequential hermaphrodite reef fishes. I provide a model that accounts for this apparent contradiction and conclude that the Gilthead seabream remains true to the size-advantage hypothesis of sex allocation theory, if size and dominance are seen as proximate, rather than ultimate, factors.

BEHAVIORAL AND NEUROENDOCRINE CORRELATES OF SEX CHANGE IN THE GILTHEAD SEABREAM, *SPARUS AURATA*.

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Professor Yonathan Zohar, Chair
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PREFACE

In 1983, when I was seven years old, all the kids in my school had either an Atari or a Nintendo. Alas, I asked my dad for one too. My father, a lawyer with an associate's in electrical engineering, was completely opposed to the idea, because he believed that those consoles were just toys. Instead, he said that if I applied myself better in school (I kept getting notes from the teachers saying I was always lost in "my own world"), that he would buy a computer for me. It was his opinion that the computer was the educational tool of the future, yet I could still play some games in it too. That year, I "earned" my first computer, a Tandy MC-10. It did not come with any games, thus I would be compelled to write my own "games". Since I was not able to understand English yet, my mom and dad would translate from the book it came with, to teach me BASIC. Four years later, I got another computer. Once again, no games came with it. But by that time, I was more interested in programming than gaming. When I graduated high school, I had already mastered several computer languages (all self-taught), and knew more about computers than my father. In the process, I had increased my English vocabulary, mastered trigonometry years ahead of my peers, and had a good understanding of the fundamentals of electrical, electronic, and computer engineering. To this date, I still think my dad is one of the smartest people I have ever known. As I grow older now, I understand that my father not only was a visionary, and made a prudent choice in offering me a computer instead of a gaming console, he went one step ahead: he gave me the tools to become a self-taught –anything-. Thanks Dad, for seeding and nurturing in me the thirst, and love, for knowledge. This thesis shows that my passion for programming is still alive after so many years and that my thirst for knowledge and learning is a flame still ablaze.

DEDICATION

For my grandfather, who died in 2008, a year before this work was completed. I miss you. You dedicated your life to raising your family out of poverty, and you told me that seeing your daughter, grandsons and granddaughters, all become college graduates, made you feel fulfilled. It only makes sense then, that I dedicate this work to you. I am proud to have known you, and even more proud to be your grandson. I hope you are proud of me too.

And also for my Mom and Dad, who have always stood with me in everything, supporting me 100% even when I thought I would not make it. While I think my dad gave me the love for learning, it was my mother who gave me the love for writing, reading, and public speaking. Each of these skills is an invaluable asset to a scientist, and thus this work is also their work. More than simply dedicating it to them, I would like to share my work with my parents.

ACKNOWLEDGEMENTS

This journey has been a long and arduous one. I started in this PhD program in August, 2001. One month later, the whole world seemed to stand still, as the Twin Towers in Manhattan fell down on September 11, 2001. That date will live forever in infamy, yet for me it marked the beginning of a new adventure, as I began my first academic year at the University of Maryland. But just as many see a disjuncture at that point in time, a pre- and post- 2001 era, I also think of my life in the same way, before and after that year. I grew up much, between that time and now, and I have too many people to thank for their help and invaluable contributions to this work.

First I want to thank my advisor, Dr. Yonathan Zohar, for providing me with great freedom to pursue my scientific curiosity and for allowing me to incorporate my other great passion, computer programming, into my work. His ability to understand my, sometimes disjointed, written "thoughts", and his great insights into fish biology and neuroendocrinology, allowed me to take this project from an idea to a "doable" work.

I also have to extend a note of thanks to all the members of my committee for providing invaluable feedback, and for taking the time off their busy schedules to join me and Yoni in the committee meetings: Dr. Frank Hanson, Dr. Greg Ball, Dr. Mary-Ann Ottinger, Dr. Andrea Johnson, and Dr. Rosemary Jagus.

I also have to thank the amazing and intelligent, then-PhD-student, who taught me (with great humility) all I know about cloning and PCRs: Dr. Ten-Tsao Wong. His friendship, great advice, good sense of humor, and keen scientific mind, helped me accomplish my goals. Thanks also to John Stubblefield, who proofread many of my earlier

works (and told me the difference between “where” and “were”), and also proofread and provided valuable feedback in the practice sessions of most of my Powerpoint presentations. Also, I can’t count the number of times I went to him with a bureaucratic problem and he calmly suggested one or two possible solutions.

I would also like to mention Dr. Frank Hanson, one of the first members in my advisory committee, who helped me get a start as an Animal Behavior scientist by providing me with good advice and experimental ideas.

All of this work would not have been possible without the help of the LMRSCS program from NOAA, which provided the funds for most of my tenure, and Dr. Rose Jagus, who oversees the program at COMB. She constantly kept pushing me to get my act together, so that I would finish, yet she made it clear that she understood all the issues that we minorities face here in the USA. She is a great asset to COMB and to the LMRCSC program.

There is a long list of people I need to say thank you, many of which did not stay to see the finished product, but whose help was invaluable to my work: Eric, Steve, Jorge, Chris, James, Joy, and all those wonderful and hard working people at ARC, Yosi Tal (a great loss to COMB), Nilli, Wei, Kathy, Ulli, Eythan, Odie, Dr. Du, and Dr. Brent Whitaker.

As a graduate student, I choose to sacrifice my social life in order to achieve my goal. However, the love and support of my partner and his family, who adopted me (First, Jon and now George), my family (Mom, Dad, and my sister) and my friends, more than made up for the lack of a it. They helped me achieve sanity during many critical moments in my life, and helped soothe the feeling of separation and loneliness that ensues in all of us, Puertorricans, when we live far from the shores of our beloved little Island.

Table of Contents

List of Tables

List of Figures

Abbreviations

AI	Artificial Intelligence
AVT/AVP	Arginine Vasotocin/Vasopressin
BAS	Background and Shadow [filter]
BNC	B Type N Connector
CC	Color Change
CRF	Corticotroping Releasing Factor
CSV	Comma Separated Values [file]
E_2	
ESTM	Expected Reproductive Success Threshold Model
GnRH	Gonadotropin Releasing Hormone
HH	Head-to-Head (same as frontal)
HPG	Hypothalamus Pituitary Gonad [Axis]
ICC	Immunocytochemistry
LH	Lutenizing Hormone
OM	Open-mouth (same as nip)
PCR	Polymarase Chain Reaction
PIT	Passive Integrated Transponders
POA	Preoptic area [of the Hypothalamus]
RGB	Red-Green-Blue
RT-qPCR	Real Time Quantitative PCR
SAH	Sex Allocation Theory
T	Testosterone
TN	Terminal Nerve

General Introduction

Study Relevance

In mammals the process of sex *differentiation* occurs early in the embryonic stage and is solely under genetic control. In fish, sex *determination* can occur in more advanced development stages and can be influenced by environmental conditions (Review: Devlin and Nagahama, 2002). The most radical form of environmental sex determination is observed in reef-dwelling species, deep water fish and in pelagic species in which adults retain the ability to change sex in response to environmental changes, even after possessing a fully functional and mature gonad of the opposite sex. The ecological and evolutionary aspects of this phenomenon, known as *sequential hermaphroditism,* have been well established (Review: Munday et al., 2006). The molecular and endocrine aspects of sex-change have also been extensively studied in many species (e.g. Wu et al., 2005). However, the way in which external cues trigger sex change is not fully understood (Godwin et al., 2003).

Two types of serial hermaphroditism exist in fish. Protogynous hermaphrodites first reproduce as females, but can change sex to male. Any male in the population comes from a previously spawning female. Similarly, in protandrous hermaphrodites, all fish first function as males but can change sex to become females. Hermaphroditism seems to have arisen in fish independently several times, and some families of fish have both types of hermaphroditism represented (Mank et a., 2006). However, protogynous hermaphroditism seems to be the most common form.

The following thesis will attempt to elucidate if social behavior can serve as the sole external cue that drives sex change in the Gilthead seabream, *Sparus aurata*

(Sparidae), a protandrous hermaphrodite. Similar studies, which correlated sex change with behavior, have focused on species that are protogynous hermaphrodites, including various species of wrasses. However, because hermaphroditism has evolved multiple times in the Teleost lineage, basic differences between protogynous sex change and protandrous sex change could be expected. In fact, among the Sparidae, some species are gonochores (e.g. *Dentex sp)*, many are protogynous, while others such as the gilthead seabream are protandrous. Protandry however, is not common among food fish and important marine stocks such as groupers are protogynous, leading to more interest in female-first sex change (e.g. Alonzo et al., 2008; Brooks et al., 2008; Hamilton et al., 2007).

Reef species have become the *de facto* model species to study how behavior influences hermaphroditism. The reef fish that became a model species for protandry is the marine clownfish, *Amphripion sp* (Pomacentridae), and it was the first protandrous species in which manipulation of social environment was shown to affect sex change (Fricke and Fricke, 1977). Several species from the wrasse group (Labridae) have been used as models for protogynous hermaphroditism, most notably *Thalassoma dupery*, since Robertson (1972) showed that manipulation of the social environment could affect sex change. Bidirectional hermaphroditism has been documented in the gobies (Warren, 1983). However, there is nothing in common between the life histories and reproductive strategies of clownfish, wrasses, gobies, and the Gilthead seabream (**Table 1**). Each of these species has variable degrees of sensitivity to social cues, as well as differences in the time it takes for the first display of behavioral sex change and gonad sex change, once

the fish has committed to sex change. Thus, extrapolating the findings from any of the research done in these species to the Gilthead seabream might not be possible.

Species	Spawning Frequency	Mating Style	Eggs	Conspicuous Sexual Dimorphism Characters/Behavior	Sex-Change
Clownfish	Protracted	Monoandry-Monogamous	Dermersal	No/Yes	Protoandrous
Wrasses	Seasonal	Diandry-Polygynous	Pelagic	Yes/Yes	Protogynous
Goby	?	Diandry-Paired with Cuckoldry	Dermersal	Yes/Yes	Protogynous (bidirectional)
Seabream	Seasonal asynchronous	Monoandry-Polyandric	Pelagic	No/Unknown	Protoandrous

Table 1. *Comparison between the life histories and reproductive strategies of the three major model species most often found in the hermaphrodite literature and those of the Gilthead seabream, the model species that is the focus of this work.*

In fact, because protandry evolved independently from protogyny, the social and biological factors that underlie the control of sex change in protogynous fish may not be the same for protandrous species. Each species may have evolved hormonal or social controls that are specific to their environmental and developmental constraints present in the ecological niche they occupy. For example, the limited social contact between conspesifics that may happen during migration in Gilthead seabream, may preclude social behavior to act as the ultimate cue for sex change. This thesis will directly address this question.

In this thesis I present the relevant findings of my work with seabream behavior. First, techniques developed to measure behavior, fish color, and other aspects of social structure will be overviewed. Chapter 2 is an in-depth study of seabream social behavior, spawning behavior and other aspects of the captive behavior of this fish. In this chapter, I will present evidence of the formation of dominance hierarchies in this species, and I will

describe, for the first time, the normal behavior repertoire of seabream, the importance of skin color as a signal of submission and aggression, and the way in which gonad development seems to influence the formation of social structures, such as dominance hierarchies, in seabream. In Chapter 3, I explore the hormones that could be involved in sex change, and the neuroendocrine factors in the brain that could be differentially expressed among sexes. In Chapter 4, I will show how specific hormones that are often linked to aggressive behavior, reproduction, spawning behaviors or sex change itself, could be influenced by social status. Finally, the correlation between dominance status, social behavior and the outcome of sex change will be reported.

The Gilthead Seabream Model

The Gilthead seabream is commonly found along the Mediterranean Sea and the eastern Atlantic Coast. Adult seabream spend most of the year in shallow coastal lagoons, but when winter approaches mature fish begin migrating towards the open sea into deeper water to spawn (Sanchez-Lamadrid, 2004). Spawning occurs between January and March, and the pelagic eggs are released into the sea (Zohar, 1978). During the spring and summer, juveniles migrate to protected coastal waters, and finally they travel back into lagoons and shallow water, where they form schools and feed on benthic mollusk (Sanchez-Lamadrid, 2004).

Major efforts to breed and mass-produce the Gilthead seabream finally succeeded in the 1980's (Moretti et al., 1999) Seabream proved to be a fast growing species, while still commanding a relatively high price in the market, and soon was aquacultured throughout much of the Mediterranean coast. Currently, it is a major aquaculture commodity in Europe. Many aspects of the gonad cycle were discovered only as part of

the effort to culture the fish. As a model species, seabream is very useful because many aspects of its endocrinology and biology have been elucidated in an effort to perfect the rearing and breeding technologies.

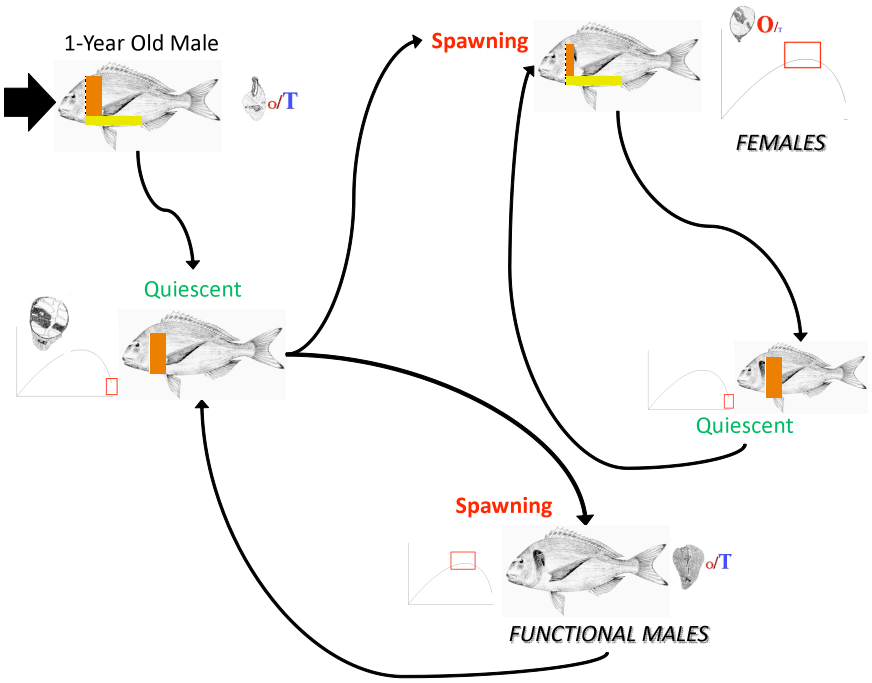


Figure 1. *The complete gonad cycle of the Gilthead seabream. Gonad development and Estradiol levels are shown in the inserts. The intensity of the pre-operculum orange color and the abdominal yellow band are also represented. At the beginning of the cycle picture (arrow), the fish are almost done with their first spawning and are functional males. Gonad diagrams and gonad pictures taken from Zohar et al. (1984).*

Original data obtained from wild populations of seabream had shown that it is a protandrous hermaphrodite (D'Anconca, 1941). A complete and detailed histological description of the gonad cycle by Zohar et al. (1984) in captive seabream provided even stronger evidence that the fish is indeed a serial protandrous hermaphrodite in which the female is the terminal sex. The detailed descriptions of the gonad cycle obtained in these studies are the basis for the description that follows.

Seabream fish develop into functional males during the first year of life. Their developing male gonad also has primordial ovarian tissue interspersed with the testicular

tissue. As the male gonad develops and becomes functional in preparation for their very first spawning season, the ovarian portion is not lost. During gonad development, a clear dorsoventral boundary becomes visible, with a ventral testicle and dorsal ovary. In the first year, this development is halted early on, and the male portion of the gonad takes over and begins development as the female portion becomes quiescent. Thus, all fish become functional males in the first spawning season (Start of cycle in **Figure 1**), but retain a small non-functioning ovary. At the end of the spawning period the gonad undergoes recrudescence. After spawning, a recrudescent ovary dominates the ambisexual gonad, but the testicular portion still remains in its ventral location.

In the next year, the gonad of these ambisexual fish begins a similar process, but in some fish the testicular portion of the gonad atrophies. In these animals the entire gonad becomes a fully functional ovary. The testicular portion of the gonad does not "re-grow" again. In the animals that retained both a male and a female gonad portion, those that functioned as males in the previous spawning season, the exact process that occurred the year before will be repeated. Thus, males will always retain the ability to spawn as females at a later time. Indeed, the terminal sex of seabream is the female sex. Once a fish becomes a female, it will not be able to spawn as a male subsequently.

Correlating to each of these periods, seabream has a gonad steroid profile (**Chapter 3**), behavioral profile (**Chapter 2**), as well as varying degrees of sex change potential (see Wong and Zohar, 2003 and **Chapter 3**). In this work, I will use the following terminology for the gonad cycle: Pre-spawning, Spawning, Post-spawning, and Quiescent. During pre-spawning, steroid gonad production has begun but not peaked, the sex of the animal is probably decided but the gonad is still developing and may still have

a male and female portion. During Spawning, the gonad is fully developed and committed, gonad steroid production peaks, and reproductive behavior is observed. During Post-Spawning, the gonad begins involution and the sex of the animal may still be apparent from the size or shape of the gonad, however gonad steroid production has declined and reproductive behavior is no longer observed or is infrequent. During the Quiescent period, the gonad is very small and consists of regressed gonad tissue, steroid production reaches its nadir (but may still produce steroids), and no reproductive behavior is observed. Molecular evidence from gonad development obtained by Wong and Zohar (2003) suggests that, during the later part of the quiescent period, gonad sex is decided. Throughout this thesis, I will discuss findings from both my neuroendocrine experiments and my behavioral observations that reinforce and expand this hypothesis.

The Evolution of Hermaphroditism

Genetic sex determination may be the ancestral state for the teleost group. This theory however remains unsupported, because the evidence is elusive (Mank et al., 2006). Regardless, fish exhibit a wide variety of alternative sex determination systems. Besides genetic determination of sex, the second most common form of sex determination is hermaphroditism. It is present across a wide variety of teleost lineages, yet it seems to have evolved independently at least 9 times (Figure 2). Among lower clade levels, where it is known that genetic sex determination is ancestral, there are still multiple instances where hermaphroditism seems to have evolved among some species. However, within the same branch, genetic sex determination may still be present in some representative of the lineage.

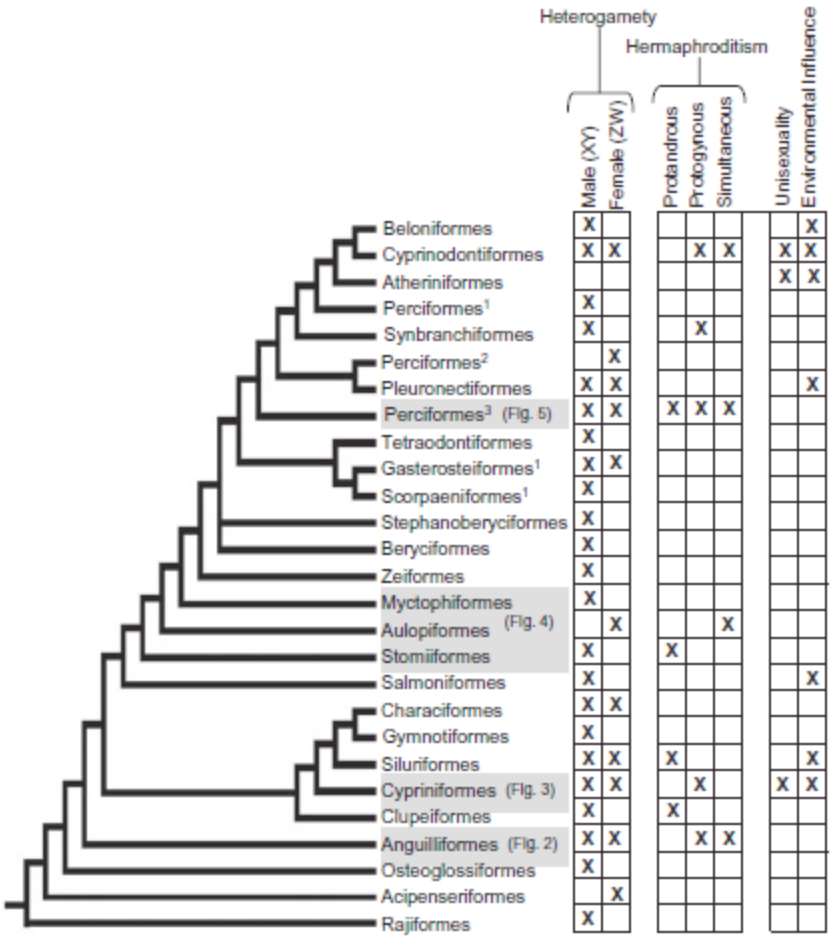


Figure 2. *Condensed, ordinal-level composite phylogeny for teleosts displaying known sex-determining mechanisms. Shown is a cladogram, adjacent to which are indicated reproductive modes recovered from the published literature. A single mark in a given row denotes that all examined species within that taxon display the indicated mode; two or more checkmarks indicate that various species within that taxon display alternative reproductive modes. Polyphyletic clades within the current taxonomy are marked and indicated to the side. Shaded boxes indicate clades that are examined in greater detail in Figs 2–5. Polyphyletic orders are indicated on the cladogram, and are as follows: Perciformes [1], Gobiesocoidei and Blennioidei; Perciformes, Caragnidae; Perciformes, Gobioidei; Gasterosteiformes, Gasterosteioidei; Scorpaeniformes, Cottoidei. (TAKEN FROM MANK ET AL. 2006)*

There is a patchy distribution of all the different sex determination mechanisms among the different lineages (Mank et al. 2006). However, reef fish are overrepresented among the hermaphrodites. The reef environment is one of patchy distribution of resources, and marine larvae can settle far apart from each other, which might be a problem during spawning even if the fish can migrate together or use visual and olfactory

cues to find a mate. Additionally, reef fish have numerous natural predators. Low population densities in the reef seem to drive the evolution of hermaphroditism in this group. This last reason might apply to deep water species too, as low population densities among deep water fish might explain the existence of hermaphroditism in many species that occupy this niche. However, it can't explain the fact that some gonochores from closely related groups are also found in deep water. Thus, although the evolutionary drive to maintain hermaphroditism might be understood, we still do not understand how or why it has evolved so many times in fishes.

Why is sex determination so flexible in fish? It is possible that because in fish both ovary and testes develop from the same precursor tissue, the potential for hermaphroditism already exists in the developing gonad (Wong, 2003). Also, in many fish the development of the gonad is not completed when the fish is born, as opposed to mammals where the process begins and ends *in utero*. The decoupling of gonad and brain development gives rise to the possibility of environmental influences on sex determination and eventually, under evolutionary pressure, might have led to the multiple appearance of sequential hermaphroditism.

From a classic Darwinian framework, sequential hermaphroditism represented a major challenge to evolutionary theory until Ghilsein (1969) extended Darwin's sexual selection theories to include hermaphroditism by introducing the Size-Advantage Hypothesis or SAH (Ghilsein, 2005). The size-advantage hypothesis is a corollary of sex allocation theory (Charnov, 1982). It predicts when an animal will change sex (Allsop and West, 2004; Allsop and West, 2003; Ghiselin, 1969). In general, the hypothesis states that sex change occurs in species where an individual is more successful

reproducing as one sex when young or small than as the same sex when old or big (Review: Munday et al., 2006). As more animals have been found that conform to the SAH model, the evidence for sex-allocation by size in hermaphroditic fish seems overwhelming. Of interest however is a recent publication by Muñoz and Warner (2004), in which they propose a modified version they termed the Expected Reproductive Success Threshold Model or ESTM (Muñoz and Warner, 2003). This model allows for females to defer sex-change to smaller females, which produces a skew in the size-distribution that can't be explained by the Size Advantage Hypothesis. Such skews are actually observed in some populations of protogynous species, which suggest that ESTM is predictive (Muñoz and Warner, 2004). However, in a discussion of sex-change in simultaneous hermaphrodites, Grober and Rodgers (2008) point out to the "overreliance" on size as a cue in the analysis of sex change, stating that: "..over-reliance on reductive cues like size… has constrained our thinking about the evolution of sexual plasticity and supports the continued publication of papers (e.g., Muñoz and Warner, 2003, 2004) that disregard… how animals make decisions about both 'gender role' and sexual allocation."

When considering how Gilthead seabream might fit into these models, it should be pointed out that protogynous hermaphroditism is more common, even among the Sparidae. ESTM has only been tested in protogynous species (e.g. Muñoz and Warner, 2004). Furthermore, most direct evidence for SAH comes from protogynous species, although a meta-analysis by Alsop and West (2003) seems to have shown that SAH can explain for all sex change modalities. Problems have been raised about their methodology (Cipriani and Collin, 2005), but their original work seems to have been validated by work from other groups (e.g. Linder and Palmer, 2008). Interestingly,

although SAH predicts a female-bias in populations of protogynous fish and a male-bias in populations of protandrous fish, the bias was stronger for protogynous species than for protandrous species, perhaps a result of unknown ecological constraints in these species (Alsop and West, 2004).

In the protandrous clownfish, evidence for SAH is very strong (Fricke and Fricke, 1977). Can we relate these findings to seabream? Clownfish females always pair with a single male. While a group of undeveloped males may remain living with the pair, they do not actively participate in spawning. In seabream, wild spawning remains undocumented but captive spawning has been observed in hatcheries (e.g. **Chapter 2**) and it seems that two males may chase an ovulating female. Furthermore, the ideal ratio for spawning seabream in captivity requires a high ratio of males to females. Together these observations suggest that seabream is a polyandrous spawner, a reproductive strategy very different from that of clownfish (**Table 1**).

In the wild, seabream migrate from their feeding grounds (in shallow water) towards deeper water for spawning. Some reports seem to suggest that these fishes live as part of small shoals. In fact, Sanchez-Lamadrid (2004) found that captive-reared gilthead seabream would form shoals with wild gilthead seabream, an observation that confirms that this species is highly social. Furthermore, group spawning has been observed in captive and wild populations. It should be noted that if extensive group fusion occurs during spawning, it may preclude social control of sex change, as it eliminates the evolutionary pressure to control sex ratios in the groups prior to spawning.

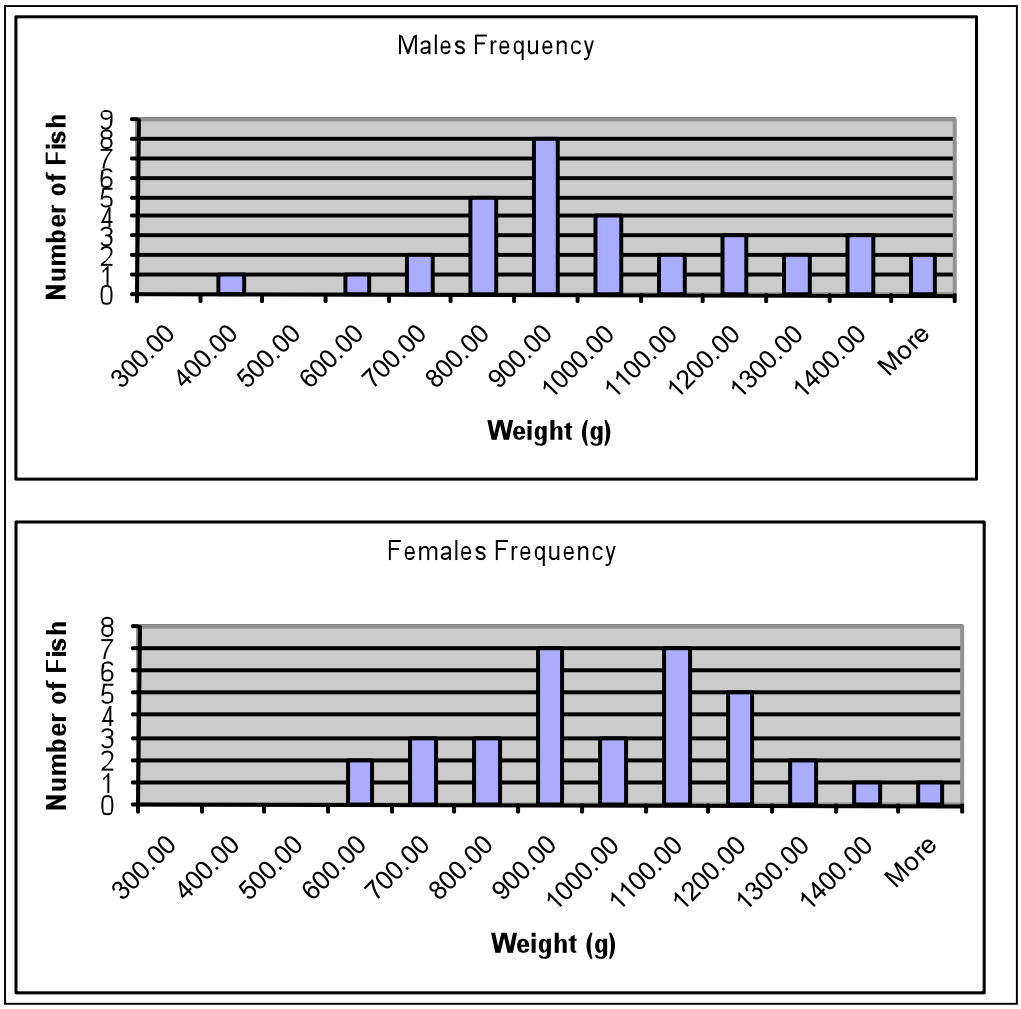


Figure 3. Histogram of weight distribution among a group of 2-year old seabream. There is a visible skew in the female sub-set of the population, suggesting a size-biased sex ratio.

Sex change in seabream can occur in isolation with inconsistent results (Wong, unpublished) and it seems that as a population of seabream broodstock ages, an overabundance of females develops (Zohar, 1988). However, in a wild population of seabream from the Mellah lagoon (Algeria), Chaoui et al. 2006 found a sex-skewed size distribution, which suggests this fish is a protandrous hermaphrodite that changes sex after reaching 44.0 cm or more. I have observed a similar skew in the weight of the animals in a 2-year old, first-time sex-changing, population kept at the PI's laboratory (**Figure 3**). However, because fish have undetermined growth, size and age could

become confounded variables. The consensus among aquaculture professionals is that seabream may change sex around some determined size, but the probability of sex change also increases with age (Zohar et al., 1990; Zohar, 1988).

As part of this work, I would like to test if the Gilthead seabream conforms better to the SAH or ESTM. In Chapter 4, I describe how size, gonadosomatic index, and other physiological factors, including dominance behaviors, may correlate with the determination of sex during the first sex change cycle of a group of seabream.

Sex Allocation Theory and Sex Change

Sex allocation theory does not explain the mechanism of sex change, but a model can be established based on its predictions. *A priori*, two explanations can be deduced: either internal (genetically programmed) signals trigger sex change, or sex change could be brought about by external factors. In shoaling or gregarious species the most likely candidate for such a "factor" is the presence of individuals of the opposite sex.

Social control may work by suppressing sex change rather than triggering it. Lorenzi et al. (2006) reported that olfactory and visual cues where not sufficient to suppress sex change in the protogynous goby *Lythypnus dalli* under conditions of social isolation or when males where separated from females with a transparent physical barrier. This result implies that physical interactions between fish are necessary to suppress sex change. It can be assumed that this requirement for physical interaction is in fact a necessity for social interaction. Dominance behavior, which requires physical interaction between the animals involved, is perhaps the most important social interaction that can affect sex change.

Dominance Hierarchies can be linked to Sex Change

Based on an earlier publication by Schjelderup-Ebbe (1922), Drews (1993) proposed a simple definition of dominance: "an attribute of the pattern of repeated, agonistic interactions between two individuals, characterized by a consistent outcome in favor of the same dyad member and a default yielding response of its opponent rather than escalation. The status of the consistent winner is dominant and that of the loser subordinate". In many animals, successive dominance interactions among different individuals lead to the formation of dominance hierarchies (Wilson, 1975), which are simple linear structures. What are the consequences for individuals organizing themselves into such social structures? Both dominant and submissive animals gain from the formation of dominance hierarchies, as was originally noted by Schjelderup-Ebbe (1935) from observing that in chickens, the pecking order succeeded in decreasing overall aggressive behavior among the members in the flock.

Dominance hierarchies do not emerge solely from dyadic interactions. Using the Siamese fighting fish, *Betta splendens,* as a model Oliveira et al. (1998) showed that by-stander fish gather information from observing conspecifics interactions. Thus, the paired interaction paradigm is not able to explain the dynamics of real and naturally occurring animal groups, because it limits the understanding of group dynamics of higher order (Earley and Dugatkin, 2002). Indeed, multi-signaler or multi-receiver interactions seem to be ubiquitous among fish, in which a complex signaling network of paired interactions are in turn affected by, and transmitted to, individuals in direct communication with the members of a dyad (Dziewecynski et al., 2005). These include the audience effect (Matos and McGregor, 2002), the context-dependent audience effect (Dziewecynski et al., 2005), and the observer effect (Oliveira et al., 1998; Johnsson and

Akerman, 1998). Another problem in dominance hierarchies is the influence of the winner-loser effect (e.g. Hsu and Wolf, 1999), which is not a multi-signaler or multi-recipient event in itself, but perhaps a combination of the effect of both types of events (observer and/or audience).

In a dominance hierarchy, the lowest ranking individuals are said to be submissive. Submissive individuals have less access to resources in the environment, including shelter, food, and spawning mates. Furthermore, the submissive individuals may show symptoms of physiological stress: higher cortisol levels are often correlated with lower social ranks (e.g. Scott and Currie, 1980). This condition is termed social stress. It is also observed in hermaphrodites. In the protandrous hermaphrodite *Amphripion sp.*, the submissive male has higher levels of cortisol when in the presence of the dominant female (Godwin, 1994). This suggests a link between social stress, dominance hierarchies and sex change. Indeed, dominance hierarchies correlate with the sex change order in spawning groups of both wrasses (Godwin et al., 1996) and clownfish (Fricke and Fricke, 1977).

Perry and Grober (2003) have proposed that this social stress, brought about by dominance behavior, and which increases serum cortisol can alter steroidogenetic pathways. It is this specific effect that suppresses sex change. Indeed, high serum cortisol levels are associated with suppressed reproductive function in fish, as has been shown for the African cichlid *Haplochromis burtoni* under conditions of social dominance (Fox et al., 1997).

Taken together, these findings suggest that the dyad is an important and visible element in the formation of social groups, but more subtle social interactions account for

the range of effects observed in animal groups in the wild or in captivity. It can be surmised that dominance behavior does not need to be conspicuously directed towards each fish in the group to have an effect on stress. For example, in a group of fish observing a fight, cortisol increased not just in the loosers. In conspecific acting as observers, a cortisol spike was also measured (Oliveira et al., 2001). Therefore, it is possible that control of the sex ratio in seabream groups is achieved by social interactions, even if such interactions do not seem to involve all the members in the social group. This is an expansion of what Zohar et al. (1984) have suggested in previous work, except here I make emphasis on the fact that the social interactions between individuals need not be directed towards the sex changing animals (i.e. females inhibiting males by dominance behavior), but that the act of dominance itself, regardless of the recipient, can suppress sex change in the males. One of the objectives of this work is to test if such a mechanism exists in seabream and if it can affect the outcome of sex change.

An Animal's Color is a Sign of Dominance Status

Body color polymorphisms are common among lower vertebrates. In fish, body coloration can be a sexually dimorphic character. However, other more labile mechanisms can also contribute to color polymorphism. For example, dominance and body color or color intensity are strongly correlated in some fish species (Korzan et al., 2008; Hoglund et al., 2000). Social stress can trigger noradrenergic pathways that lead to physiologic color changes (Review: Sugimoto, 2002). This type of color change is transient, but can become morphological or permanent under the certain conditions. For example, physiologic color changes that occur under stress can become permanent color morphs because stress-induced submissiveness is associated with changes in the size and number of brain pre-opiomelanocorticotropin hormone (POMC) neurons (Hoglund et al.,

2000), the precursor of α -melanocyte stimulating hormone (α-MSH). This hormone can also act peripherally to induce permanent color changes (Review: Kawauchi and Baker, 2004). The existence of such mechanisms may explain why in artic feces, *Salvelinus alpinus*, intensity of skin color can be correlated to social rank (Hoglund, 2000). Color changes can be part of specific social signals involved in agonistic interactions in fish (Barlow, 1963; Hurd, 1997). Rapid but transient color changes have been observed in seabream during escalated aggression and during spawning (**Chapter 2**). These behaviors are probably associated with the establishment of territories and may be a communication signal that conveys readiness to spawn or engage in aggressive bouts.

Like with other aspects of behavior, the relationship between color and aggression can be reciprocal. Growing red porgy in dark backgrounds increases cortisol levels in the blood when compared to fish grown on white backgrounds (Rotllant et al., 2003). In artic feces, white background increases aggression (Hoglund, 2002). Such reciprocal relationship suggests that color change may be a pervasive and important signaling modality in fish that conveys the individual's motivation to fight.

In lizards, the operculum color can affect the perceived dominance of an individual, and manipulating this signal can change the status of the individual, as well as the opponent's motivation to fight (Korzan and Summers, 2004). Currently, the significance of color changing behavior as a social signal in seabream is unknown. Since this signal could play a role in establishing dominance, and communicating social or reproductive status, a comprehensive study of seabream behavior and its role in sex change needs to address the role of both physiologic and morphologic color changes within their behavioral contexts.

Dominance and Availability of Food Resources Affects Reproductive Status by Influencing the HPG axis

The effects of social stress on fish are not confined to aggressive behavior or skin color. For many years, farmers and scientist have known that stress itself can have an effect on gonad size and reproductive status, perhaps by acting on the hypothalamus-pituitary-gonad axis itself (Fox et al., 1997; Selye, 1946). Social stress can also affect reproductive behavior, an effect that can be dependent on the specific stressor (Retana-Marquez et al., 2003). In vertebrates, the hypothalamus, the pituitary gland and the gonads (HPG) form an axis that controls not only gonad maturation and reproductive function, but male-typical and female-typical reproductive behaviors (Porterfield, 1996). By affecting the HPG axis, social stress, and in a broader sense any type of social behavior, can affect the process of gonad maturation, reproductive status, reproductive behavior and sex change outcome.

Food availability also has a direct effect on reproduction and gonad maturation. The rank of each fish in a dominance hierarchy may determine its ability to access food resources in the environment. Indeed, social status regulates growth rate (Buston, 2003; Hofmann et al., 1999). Buston (2003) has posited that perhaps such regulation maintains relative size differences in social groups. These size differences may help eliminate the need to use aggressive behaviors as a means of establishing dominance. Thus, because fish size and age may determine the rank of an individual within a hierarchy, the two factors may also be interdependent or covariates in this context too. The Size-advantage Hypothesis, predicts that factors affecting allocation of resources also affect sex determination. Hence, the relationship between appetite and the HPG axis is important to address here, albeit if only briefly.

The Role of the GnRHs in the Control of Reproductive Status

In teleost fish, the main regulator of the HPG axis is Gonadotropin Releasing Hormone (GnRH). It is the endogenous releaser of gonadotropins in all vertebrates (Review: Zohar et. al, *in press*). In fish, it is secreted into the pituitary by direct innervation from cells in the hypothalamus, while in mammals a portal circuit delivers GnRH to the pituitary gland from the hypothalamus (Review : Foran and Bass, 1999). GnRH initiates the hormonal cascade that leads to the onset of gonadal maturation, including stimulating the secretion of

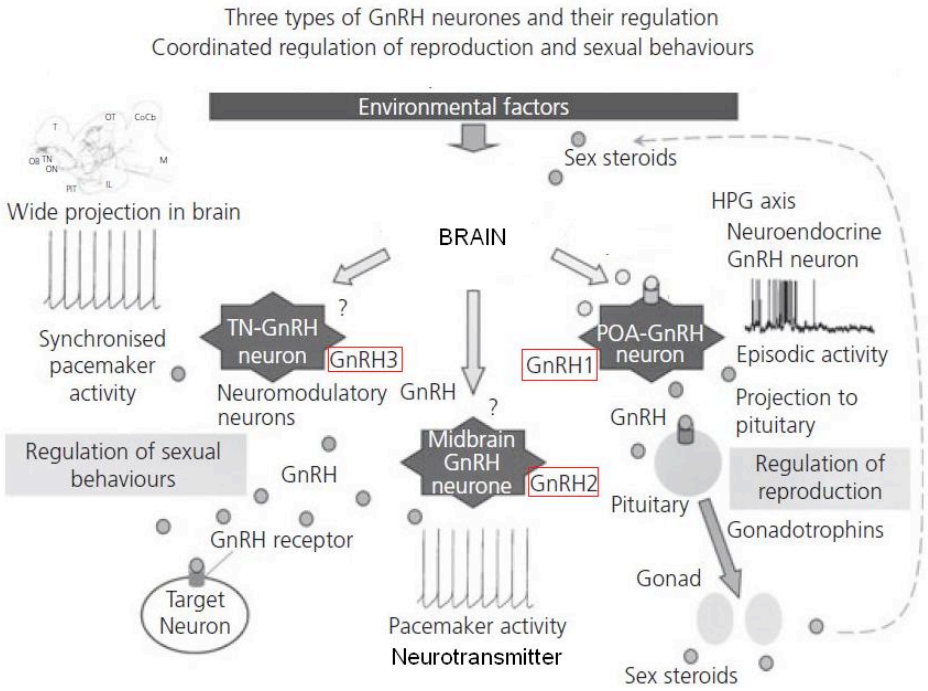


Figure 4. Functional-based nomenclature of the GnRH system and the phylogenetic-based equivalent in seabream. GnRH-1 is located in the preoptic area of the hypothalamus. GnRH-2 is located in the midbrain region and might be a ubiquitous neurotransmitter. GnRH-3 is usually found in the terminal nerve. Figure from Oka (2009).

Lutenizing Hormone (LH). It is also involved in other aspects of reproduction, including behavior (Porterfield, 1996). GnRH is the first step in the activation of the HPG axis. I

have already defined several factors that may affect sex change: social stress, aggressive/submissive interactions, and food availability. All of these factors have been shown to affect the GnRH system. Therefore, the HPG axis itself may play a central role in the transduction of the environmental signals that lead to sex change.

To understand the role of GnRH in reproduction, the multiplicity of GnRH has to be addressed first. Multiple forms of GnRH have evolved in different species, and many species carry genes that code more than one form of the GnRH peptide (Review: Zohar et al., 2009). It is possible that they arose from gene duplication (Okubo and Nagahama, 2008). Each of the forms discovered in different species can be classified by the gene family they belong to, because each isoform is coded by a different gene (Fernald and White, 1999). It is thought that each GnRH form or gene family may have different and highly specific functions in the control of fish reproduction (Weltzien et al., 2004), because each isoform is present in a different region of the brain (Gothilf et al., 1995). While most species studied have at least two GnRH forms, many teleost, including seabream, have a third form of GnRH (Powell et al., 1994). Until the discovery of this third form, it was thought that GnRH-1 was confined to the anterior part of the brain, while GnRH-2 was expressed usually in the posterior part of the brain (Review: Zohar et al., 2009). The discovery of a third form within the anterior region of the brain lead to a new paradigm and subsequently, to the idea that each of the 3 GnRH's is highly localized to different regions of the brain. In species with 3 GnRH's, GnRH-1 is the hypophysiotropic form. In this fish, such as seabream, GnRH-1 is found in the hypothalamus (Parhar, 2002), while GnRH-2 is distributed throughout the brain (Zohar et

al., 1995). GnRH-3, may be found in the olfactory bulb of some species (Amano et al., 1991).

Fernald and White (1999) proposed that the phylogeny of the GnRH system be addressed so as to name each form depending on their phylogenic relationship to the human GnRH-1. Each of the 3 gene families correspond to the 3 GnRH's that have been isolated from seabream. This might not be the case in species that have two GnRH forms (such as salmonids and zebrafish). In those species, the function of GnRH-1 (hypophysiotropic) is now taken over by the GnRH-3 orthologue of that species (Review: Zohar et al., 2009). In fact, GnRH-3 neurons are found in the preoptic area and the olfactory bulb of zebrafish, thus fulfilling both the function and localization associated with GnRH-1 and GnRH-3 in the "3-form" fishes (Abraham et al., 2009).

These recent findings have led to an even greater paradigm shift because one form of GnRH in one fish can perform a different function in another species. Thus, a nomenclature based in *function* is now being advocated, while still preserving the phylogenetic nomenclature (e.g. Oka, 2009). Under this new paradigm (**Figure 4**), GnRH-1 in seabream becomes the POA-GnRH, because it is located in the preoptic area of the hypothalamus (Gothilf et al., 1996). GnRH-2 is the midbrain GnRH, controlling behaviors associated with reproduction (Hoskias et al., 2008, Kauffman and Rissman, 2004; Temple et al., 2003; White, 1995). This second form is not believed to be directly involved in release of LH or gonadal development in fish with 3 GnRH's (Gault et al., 2003). As noted above, seabream has a third GnRH form, the Terminal Nerve-GnRH or TN-GnRH. In seabream this is GnRH-3. It is found in the terminal nerve region of teleost species that posses it, and probably modulates visual or olfactory information

relevant to reproductive behavior (Zhang and Delay, 2007; Grens et al., 2005; Wielchmann and Wielchmann, 2001; Yamamoto and Kawashima, 1997). GnRH-3 probably evolved from GnRH-1, and it could be the most evolutionary-derived form (Fernald and White, 1999). GnRH-3 may also have a role beyond affecting sensory pathways, such as modulating reproductive behavior *per se* (Ogawa et al., 2006).

Social stress downregulates GnRH-1 and GnRH-2 mRNA levels in the brain of tilapia (Ogawa et al., 2003). In mammals, a GnRH agonist has anxiolytic effects that antagonize the role of the anxiogenic peptide CRF (Umathe et al., 2008). Dominance hierarchies, which probably play a role in social stress, also affect the GnRH system. For example, the size of hypothalamic GnRH cells is reduced in subordinate fish (Fox et al., 1997). These changes in cell size might be related to changes in production of GnRH. For example, in male tilapia forced to "descend" in social rank, an increase in plasma cortisol was accompanied by an increase in brain GnRH-1 mRNA transcripts (Parikh, 2006).

In clownfish the number of GnRH cells in the Pre-Optic Area (POA) of the hypothalamus is higher in the submissive male when compared to a female of similar weight (Elofsson et al., 1997). In the ballan wrasse, *Labrus berggylta* (Labridae), the more dominant male had higher numbers of GnRH cells when compared to females of similar weight (Elofsson et al., 1999).

Orexin, an appetite-inducing hormone, and GnRH have been shown to regulate feeding behavior in the goldfish, *Carassius aruatus* (Cyprinidae), (Hoskins et al., 2008). In the Japanese quail, the induction of LH secretion by GnRH can be attenuated by caloric restrictive diets (Ottinger et al., 2005). The role that different GnRH isoforms

play in the expression of behavior and how environmental factors mediate their effects in the HPG axis by modulating the expression of these isoforms in different regions of the brain, was reviewed recently by Hoffman (2006).

AVT Mediates Aggressive Behavior and may be Involved in Dominance

As the activator of the HPG axis, GnRH may be the most important neuroendocrine factor controlling reproductive function. However, control of reproductive behavior is complex, and several factors interact with the HPG axis to regulate these behaviors. One of these factors is a small nonapeptide that has remained highly conserved among all the vertebrates: Arginine Vasopressin (AVP), a hormone that acts as vasopressor and stress hormone in higher vertebrates (Review: Rose and Moore, 2002). In humans it is known as anti-diuretic hormone (ADH), because of its role in controlling water balance (Porterfield, 1996). It can increase adrenal cortisol secretion by increasing the sensitivity of corticotropes to CRH (Porterfield, 1996).

The teleost analogue to AVP is Arginine Vasotocin (AVT). Evidence from experiments in birds, reptiles and fish suggest that AVT is involved in the control of reproductive and social behaviors (Review: Insel and Young, 2000). It probably plays a role in spatial memory and space-dependent behaviors (e.g., territorial aggression) by acting as a neuromodulator of sensory and motor pathways (Rose and Moore, 2002; Ferguson et al., 2002).

Anatomical studies of the vasotocin POA neurons in fish, suggest that these neurons organize in cell-type specific clusters and that they send outputs to the pituitary, the ventral telencephalon and ventral thalamus (Saito et al., 2004). Thus, like GnRH,

AVT cells send simultaneous projections to both the pituitary and the telencephalon, which suggests that they could act in concert to modulate behavior.

Pickford and Strecker (1977) showed that AVT could trigger the spawning reflex in killifishes. Later it was shown that AVT is involved in the control of reproductive behavior in many other fish species (Review: Foran and Bass, 1999). Indeed, there is evidence that this function of AVT is conserved among the vertebrates, including amphibians (Ten Eyck, 2005; Marler et al., 1999; Propper and Dixon, 1997), birds (Jurkevich et al., 2001; Goodson, 1998) and hermaphrodite fish species (Grober et al., 2002; Godwin et al., 2000; Foran and Bass, 1998; Grober and Sunobe, 1996).

The behaviors that AVT seems to induce in fish can be very specific. For example, in white perch, *Morone americana*, attending behavior is the primary courtship ritual leading to spawning. Attending behavior is unique to males. An attending male chases the female and attempts to contact her abdomen using his snout (Salek et al., 2001). Intracerebroventricular injections of AVT increase attending behavior in the male of this fish (Salek et al., 2002).

GnRH and AVT Could Work Together to Coordinate Behavior

GnRH and AVT play important roles in the control of reproduction and sexual behavior. Each neuropeptide influences different aspects of behavior. AVT seems to play an important role in territorial aggression, affiliative behavior, and agonistic behaviors. GnRH initiates gonad development, but also regulates reproductive behavior. Because multiple isoforms of GnRH exist, it is likely that GnRH is involved in almost every aspect of reproduction. Regardless, only a handful of studies have attempted to understand the influence of each peptide system on behavioral aspects of reproduction.

Such studies are very important to understand how sex change may work because, as Foran and Bass (1999) have emphasized, hypothalamic AVT and GnRH are putative "axis of sexual plasticity" in teleost.

In amphibians, calling is an important mating behavior and amplexus is a type of amphibian mounting behavior that only occurs during mating. AVT induces calling behavior in anuran amphibians, while GnRH promotes amplexus (Propper and Dixon, 1997). The two pathways are independent, so that GnRH and AVT have differential effects in these behaviors. In the musk shrew, *Suncus murinus*, AVP increases scent marking in the female, while exogenous GnRH seems to decrease the latency to rump, present, and tail wag (Schiml and Rissman, 2000). Together, this evidence suggests that GnRH and AVT/AVP have separate roles in the control and initiation of behavior.

Anatomically, the GnRH and AVT/AVP systems have a very close relationship. For example, GnRH and AVT cells co-occur in the same region of the hypothalamus. There, usually in the Preoptic nucleus (POA), the vasopressin neurons synapse with GnRH-producing cells. The synapsing of the two systems has been shown in almost every major vertebrate group, including mammals (Thind et al., 1991), birds (D'Hondt, 2000), amphibians (Jokura and Urano, 1985), and fish (Amano et al., 1991; Bailhache, 1994). Perhaps, this is evidence of the necessity to coordinate reproduction-related behaviors.

AVP/AVT may play a role in other physiological aspects of reproduction. Thus, AVP can induce an *LH surge* in rats (Palm et al., 1999), perhaps by acting as a circadian signal (Miller et al., 2006). AVP cells of the suprachiasmatic nucleus of rats probably drive the circadian rhythm pattern of GnRH secretion from POA cells, as evidenced by *in*

vitro experiments (Funabashi et al., 2000). Thus, GnRH cells in the hypothalamus probably communicate with AVT cells, either directly or indirectly. One of the possibilities, at least in mammals, is that AVT cells may mediate the estrogen modulation of LH (D'Hondt et al., 1999). Some aspects of sex change, such as social stress, may bring together both systems: AVP has also been implicated in the stress-induced *suppression of LH* in the female rat (Cates et al., 1999).

The relationship between AVP/AVT and GnRH is not limited to the effects of AVP/AVT on the GnRH cells. It is possible that in different brain loci or for different isoforms of GnRH, the relationship is reciprocal. For example, rainbow trout has two native isoforms of GnRH: GnRH-2 and GnRH-3. When either of these two GnRH isoforms was applied *in vitro* to preparations of hemisected trout brains, the periodic calcium pulses of vasotocin neurons were shortened, a response that seems to be specifically mediated by a GnRH-receptor (Saito et al., 2004).

Thus, many questions about the relationship between specific GnRH isoforms and AVT remain unanswered. Also, these factors should be considered when designing and analyzing experiments that test the relationship between behavior and the GnRHs or AVT. In the Gilthead seabream, there are no studies that explore the relationship between these two peptide families. Furthermore, the relationship between these peptides and hermaphroditism, in protandrous pelagic fish, has never been explored.

Neurosteroids

Neuropeptides are not the only neuroendocrine factors that can control behavior. Steroids produced locally in the brain ("neurosteroids") can induce specific reproductive behaviors (Schmidt et al., 2008; Balthazhar and Ball, 2006), and can act as

neuromodulators or neurotransmitters in the brain via non-genomic effects (Remage-Healey and Bass, 2006a; Review: Ball and Balthazart, 2004). Furthermore, gonadal steroids such as testosterone are associated with aggressive behavior (Earley and Hsu, 2008), as well as social status in vertebrates (Holmes et al., 2008). Indeed, the winner/looser effect may be mediated in part by testosterone (Trainor et al, 2004; Oliveira et al., 2001). Rapid changes in circulating steroids levels may be linked to rapid changes in behavior following a fight or territorial contest (Remage-Healey and Bass, 2006b). Steroids have organizational effects on brain tissue, and in mammals they are required for the proper development of sexual behavior (Bakker et al., 2002). They are also important in the establishment of sexually dimorphic color patterns in hermaphrodite fish (Cardwell and Liley, 1991), and they play a role in the process of sex determination by directing the phenotype of sexually dimorphic regions in the brain (Review: Kudwa et al., 2006), perhaps by activating apoptotic pathways (Review: Forger, 2006). Therefore, it can be hypothesized that gonadal steroids are important in the behavioral changes that occur after sex change in seabream.

AVT stimulates the biosynthesis of neurosteroids in the brain of the frog (Do Rego et al., 2006). Recently, Bass (2008) has reviewed the role that steroids play in the rapid modulation of the firing properties of a central pattern generator, which controls vocalization in a teleost fish. Neurosteroids may play important role in behaviors even in the absence of gonadal steroids, because such behaviors precede sex change. In the bluehead wrasse ovariectomy of the dominant female prior to male removal does not preclude behavioral sex change (Godwin et al., 1996). Neither, castration of the territorial male, nor ovariectomy of the dominant female had an effect on the behavior or

the level of AVT mRNA in the brain. However, after achieving social dominance, only intact females showed an increase in the soma size of gigantocellular AVT-ir POA cells. Similarly, exogenous testosterone increases AVT expression and triggers mounting behavior in the whiptail lizard (Hillsman et al., 2007). Thus, these suggest that steroids produced by gonads are important in triggering the changes in the transcriptome that lead to brain remodeling and permanent behavior change.

In this thesis work, I will not examine the role of neurosteroids, but the potential role they may play in controlling behavior and regulating reproduction will be considered when analyzing the data obtained. In Chapter 3 and 4, I will discuss the possible relationship between GnRH, social behavior, AVT and the gonad steroid estradiol.

Project Goals

The primary goal of this research is to advance our understanding of the behavioral and hormonal mechanisms that are involved in the regulation of sex reversal in the protandrous hermaphrodite, gilthead seabream (*Sparus aurata*). To accomplish this goal we will test the following three hypotheses:

Objectives

Hypothesis 1: In captivity, seabream interact with one another to form dominance hierarchies. The stability of these hierarchies as well as the intensity of aggressive interactions between fishes in the social group will show a seasonal pattern. The seasonality of these behaviors can be attributed to different gonad stages. Color changing behavior forms part of the suites of behaviors involved in seabream dominance-aggressive interactions.

Hypothesis 2: Aggressive individuals preclude submissive fish from changing sex. Submissive individuals tend to stay as males, while aggressive individuals become females. Ultimately, control of sex ratios is achieved in a way that follows the predictions of the Size-Advantage Hypothesis.

Hypothesis 3: Social sex control is achieved largely by dominance-submissive interactions, and these behaviors are under the influence of several hormones. Among these hormones and neuropeptides, AVT, GnRH-2, and gonadal steroids, strongly influence the aggressive or submissive status of a fish by modifying the fish behavior. These factors also interact among themselves.

To support these hypotheses, I have designed a series of experiments each of which will fulfill the following experimental objectives:

1. Characterize agonistic behaviors, including aggressive, territorial and submissive behaviors throughout the gonadal cycle. (**Chapter 1 and 2)**

2. Correlate aggressive behaviors and dominance rank to gonad development, fish gonad sex, steroid levels, and the outcome of sex change. **(Chapter 3)**.

3. Clone the AVT mRNA in seabream and quantify its seasonal expression, as well as the expression levels of the 3 GnRHs in this species. Correlate AVT, GnRH, and gonadal steroids to aggressive behavior or dominance rank, and sex. **(Chapter 4)**

Chapter 1A: *AquaObserver,* a tool for the analysis and measurement of fish dominance and aggressive behavior.

Introduction

Fish show a wide range of behaviors, including aggression against conspecifics, submission to dominant individuals, posturing to defend territory or to signal submission, spawning sex-specific behaviors, and even predator avoidance behaviors. The richness of their behavioral repertoire, together with the diversity of the ecological niches they occupy and reproductive strategies they use, make fish a great model species for animal behavior studies. However, measuring behavior is not a trivial task. Although a behavior might be conspicuous (e.g. fin nipping), the factors that trigger it might not be readily known (e.g. perceived threat, hunger), or may be difficult to observe (e.g. opponent has signalled aggression intent with a conspicuous signal). Like other animal networks, fish social networks probably rely on behaviors and communication signals that can easily disperse throughout the network. The significance of some of these intraspecific communication signals, such as body coloration, might be hard to assess because of the typical experimental design limitations encountered in fish behavior studies. The importance of each of these aspects in behavior analysis varies with each species, and with each experimental design or objective. Thus, although many methods are standard in animal research literature, it is not unusual for a researcher to tailor the methodology and the analytical tools to a specific species or experimental setup. Here, I discuss the development of a suite of tools that I developed to study the captive behavior of the Gilthead seabream. Specifically, I developed a series of algorithms and mathematical tools to study certain aspects of the formation of hierarchies in captive seabream. I also

developed *de novo*, an algorithm that can track and measure fish body coloration. Both of these tools are integrated into a software package that I named *AquaObserver*. Among the unique advantages of this software is that it enables a seamless integration of postural, dyadic, and group-level metrics, which allow the researcher to have a comprehensive understanding of the many variables that affect the fish status in the group, and the dynamics of the formation of dominance hierarchies.

There are four reasons that compelled me to create these set of tools: 1) The animals had long periods of inactivity, which made it more difficult to rely on direct observations or standard video technologies; 2) The original experimental design required the animals to interact in so called embedded dyads (discussed later in this chapter) consisting of several fish to avoid social isolation. However, embedding the dyads made the calculation of ranks computationally intensive; 3) After computing rank for the first few observations performed, I realized that the rank of the animal was not a stable parameter, which then prompted me to develop a stability index, adding an additional level of complexity to the algorithm which increased the required computational effort (if done by hand); 4) Aquaculture raceways are large, with low-intensity lights, variable water quality, and an ever-changing background (due to algal growth, food detritus, etc), which made estimating fish color by a human observer, a nearly impossible task. *AquaObserver* addresses each of these problems, providing software-based computational solutions that required minimum or no modifications to the experimental protocol.

Computer software can be used to simplify the process of both analysis and data collection of behavior experiments (Martin and Bateson, 1983). Indeed, many

applications have been developed to enhance the analysis, data collection, and meta-analysis of animal and human behavior (e.g. Park et al., 2005; Kato et al., 2004; Rowley et al., 2003; Schwarz et al., 2002; Noldus et al., 2001; Noldus et al., 2000; Winberg et al., 1993).

To study dominance behavior, a researcher usually employs a method called the dyadic encounter. The term dyad, or pair, is used to underline the fact that each fish in a group is paired with another, usually in isolation, until all possible pairings are considered. This all-play-all (aka round-robin) approach yields the necessary information to evaluate the entire relationship among all group members. However, this method has one clear disadvantage: the animals interact in isolation. This isolation from the social group, precludes context-dependent interactions from occurring (Chase et al., 2002; Dzieweczynski et al., 2005). Furthermore, in shoaling fish such as seabream, social isolation might be a stressful condition which can affect the results of the experiment. Seabream do not fare well when housed singly, and even when paired with another fish, seabream might still refuse to eat for several days (Per. Obs.). This stress can potentially confound the results of the encounters.

Paired context-dependent interactions affect the formation and structure of dominance hierarchies (Oliveira et al., 1998; Chase et al., 2003). To study the formation of dominance hierarchies, the dyad should be embedded in a group (Chase et al., 2003). Such "socially embedded" dyads have recently become the focus of research into dominance behavior and dominance hierarchy formation (Sloman, 2007; Valderrabano-Ibarra et al., 2007). Indeed, Wey et al., 2008 has suggested that animal behaviorist study the behavior of animal in groups using social network analysis. The elements of a social

network have been identified in many fish species (Plath et al., 2008; Chase et al., 2003; Earley and Dugatkin, 2002) and different parameters of social networks have been measured and studied in fish (Croft et al., 2005). Recently, Pike et al. (2008) demonstrated that fish social network structure can be affected by the "behavioral composition" of the individuals in the group. New research methods and statistical analysis tools suitable for studying socially embedded dyads have been developed in the last decade (de Vries, 1998; Langbein and Puppe, 2004). Most of these were originally used in isolated dyads, but they have been extended to socially embedded pairs with some modifications (de Vries, 1998). Here, I propose a method to study hierarchy stability in socially embedded dyads using an index that is simple to calculate.

Only two methods are widely used to measure stability of the dominance hierarchies: the Spearman rank correlation (ρ), and a permutation method developed by de Vries et al. (1993) which is based on three correlation tests including Spearman's ρ, and Kendalls τ. Both Spearman and Kendall statistics were developed for testing rank correlations to assess trends or similarities between two samples. Although both these tests can be applied to rank data, de Vries (1993) applies his test to the dominance matrix.

The instability index that we developed works directly on rank data. Because the value of this index (S) increases when the hierarchy destabilizes, I decided to use the term *instability*, so that the interpretation of the calculated value is self-evident. Our method is designed to assess the instability of animal groups in which aggressive behavior occurs infrequently, a common problem with many pelagic species observed in captivity. Indeed, it is similar to a Spearman's ρ but can be applied directly to the rank data output from our ranking algorithm. Its implementation is simple, regardless of group size.

Another type of behavior that might influence the formation of hierarchies, but which is extremely difficult to quantify, is body color change. Color changing behavior is a type of posture behavior, because the animal's color might reflect its willingness to fight in response to a confrontation. It might also be characterized as a behavioral state, as the body color might also be linked to spawning status, submissive status, etc. Indeed, in fish melanic changes in body coloration are common intraspecific behavior signals. In the last part of this chapter, I will discuss a series of image analysis algorithms that allow measurement of fast color changes in actively swimming fish. The technique was developed for aquaculture raceways and works well in poor lighting conditions.

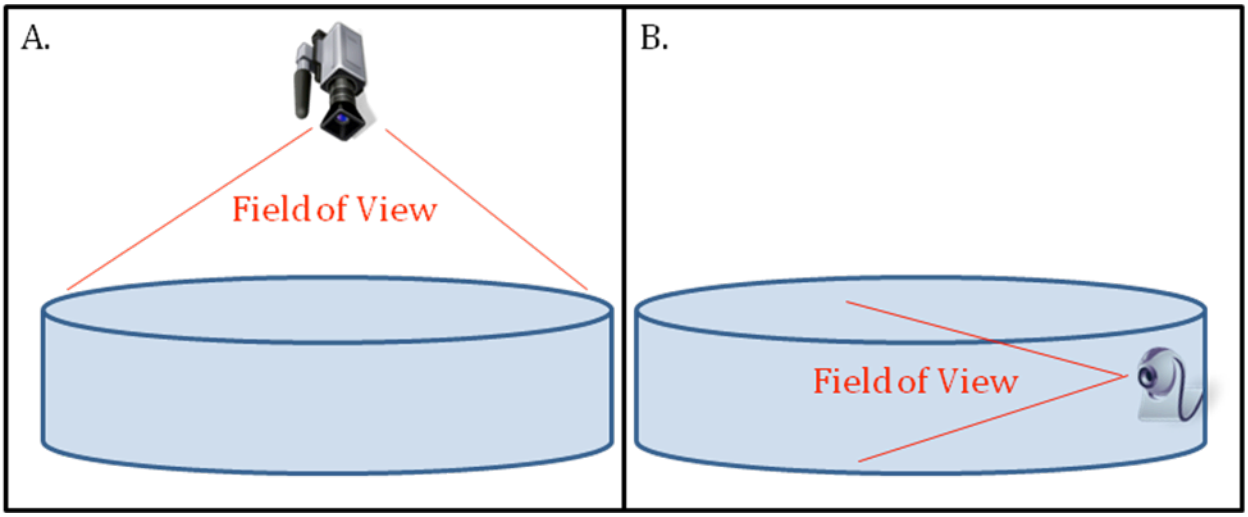


Figure 5. Filming of fish was usually accomplished by using an above-the-tank mounted camera (A), but to better define behaviors, one tank was fitted with an underwater camera system that allowed recording using a fish-eye view of the behaviors.

Definition of Behaviors

To establish the behaviors that could be scored as aggressive, I observed fish in both videotaped and real-time paired encounters, and in videotaped group interactions. Although for most of the work I present here, I used a camera angle perpendicular to the fish principal movement plane (**Figure 5a**), I fitted one of our tanks with a camera that

was placed below the water line. This camera had a field of view that did not encompass the entire tank but was parallel to the fish principal movement plane (**Figure 5b**). This "fish-eye-view" setup, and the observations of pairs that were done in real-time, allowed me to describe the behavior of the fish in more detail. Based on similar behaviors observed in Cichlids that were defined by Carruth (2000), I identified 3 main agonistic interactions: chases, nips, and frontal display. A chase occurs when a fish changes its swimming direction and speed in response to a sudden approach by another fish. A nip is defined in a similar way, but the attacker may already be in

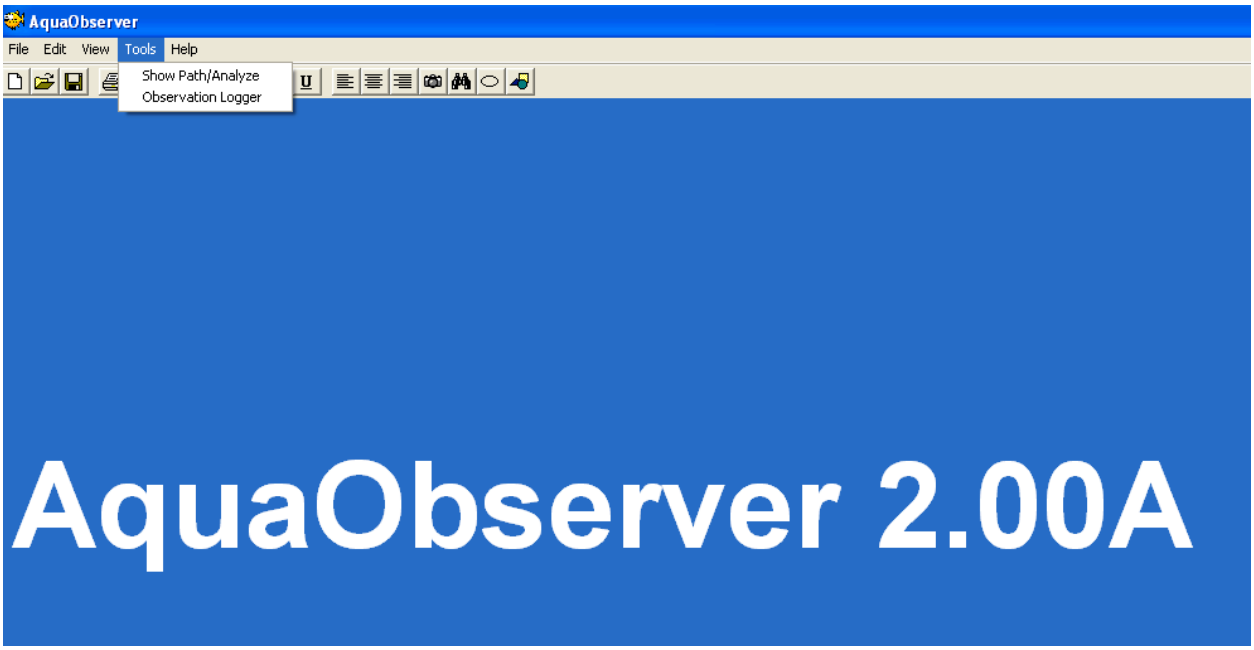


Figure 6. AquaObserver's main user interface provides access to all 3 modules: An observation logger and video player, a track and behavior analysis tool, and an image analysis and fish tracking tool.

close proximity to the attacked fish and the attacker adopts an "open mouth" posture. The frontal aggression usually precedes or follows a chase or nip, and may be a dominance display in which the fish "measure" their strength by assessing their size directly. It is characterized by an "open-mouth" posture, but both fish are in the same

posture, facing each other. This pose lasts a few seconds or less, and then one fish moves away. The attacker in a frontal aggression is defined as the fish that moves forward, while the fish that "reverses" and flees is the attacked fish. In seabream, this behavior probably represents an escalation of aggression. Similar to what Carruth (2000) reported for *Crenicara punctulata*, a protogynous cichlid, this behavior is the most infrequently observed in seabream.

***AquaObserver*: Program Description**

AquaObserver was written in Microsoft Visual Basic 6.0. It consists of three main program "modules" that share a single user interface (**Figure 6** and **Figure 7**). The first program module is an image analysis and computer vision application developed to measure fish color, study fish melanic patterns and morphs, and to track fish. The second module lets the user visualize fish movement (tracks), and analyze territorial behavior based on positional data from track analysis.

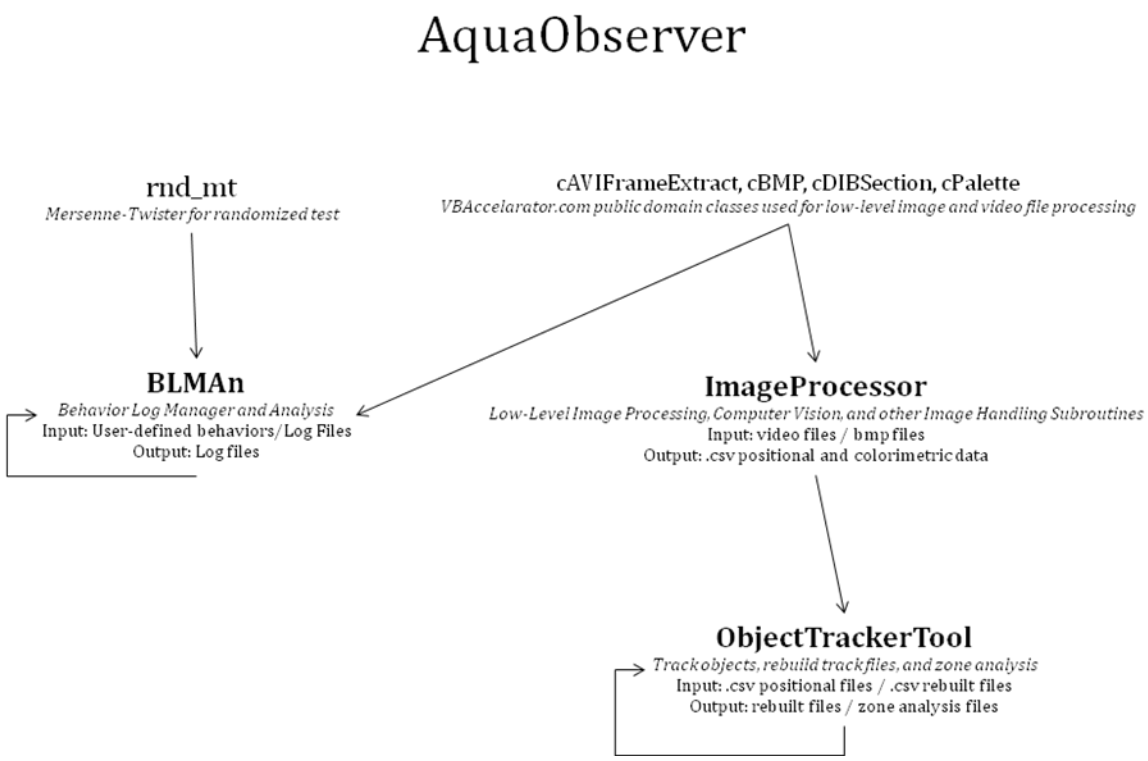


Figure 7. Flow of information between AquaObsever's 3 main modules (in boldface) and the overall program architecture. Arrows point from data source to consumer. BLMAn and ObjectTrackerTool are classes that generate data (as .CSV files or .TXT files), which they also analyze further, thus they have arrows that point back to themselves.

The third module consists of a video player and a time-stamped event logger. Using these tools, the user can watch recorded videos to create a time-stamped behavior log without having to use an external video player. The player also provides contrast and brightness controls, allowing the user to enhance the video to better visualize the animals in the arena. *AquaOberver* can further analyze the data contained in the log, by creating dominance matrices, deducing dominance order, and measuring the instability of the generated ranks. Although, the image analysis software and the behavior analysis program generate data independent of each other, the information can be merged to provide a more comprehensive analysis of fish behavior. All data generated by

AquaObserver is outputted to comma-delimited files (.CSV) files, which can be opened by Microsoft Excel or other commercially available applications.

To log stereotyped behaviors, the researcher plays back the video files using *AquaObserver* built-in video player and records the behavior in the electronic behavior log. The video player can be set to play at any speed, so that if behaviors of interest are interspersed with long periods of inactivity, the user can fast forward through them. The researcher can also instantly replay the last second of observed video in order to watch behavior that may be difficult to assess. The keyboard function keys can be programmed to log specific behaviors with one or two keystrokes. Each entry logged has a time-stamp appended to it. The time-stamp serves as a video file "bookmark", and any behavior logged can be reviewed using a user-programmable video loop. The bookmarks are useful for scanning a video file and finding behaviors of interest for further study or to validate behavioral observations performed by different observers. In addition, each log file contains a header with a table that contains detailed information about each animal in the experiment (the user can store any information, such as weight, age, sex, etc).

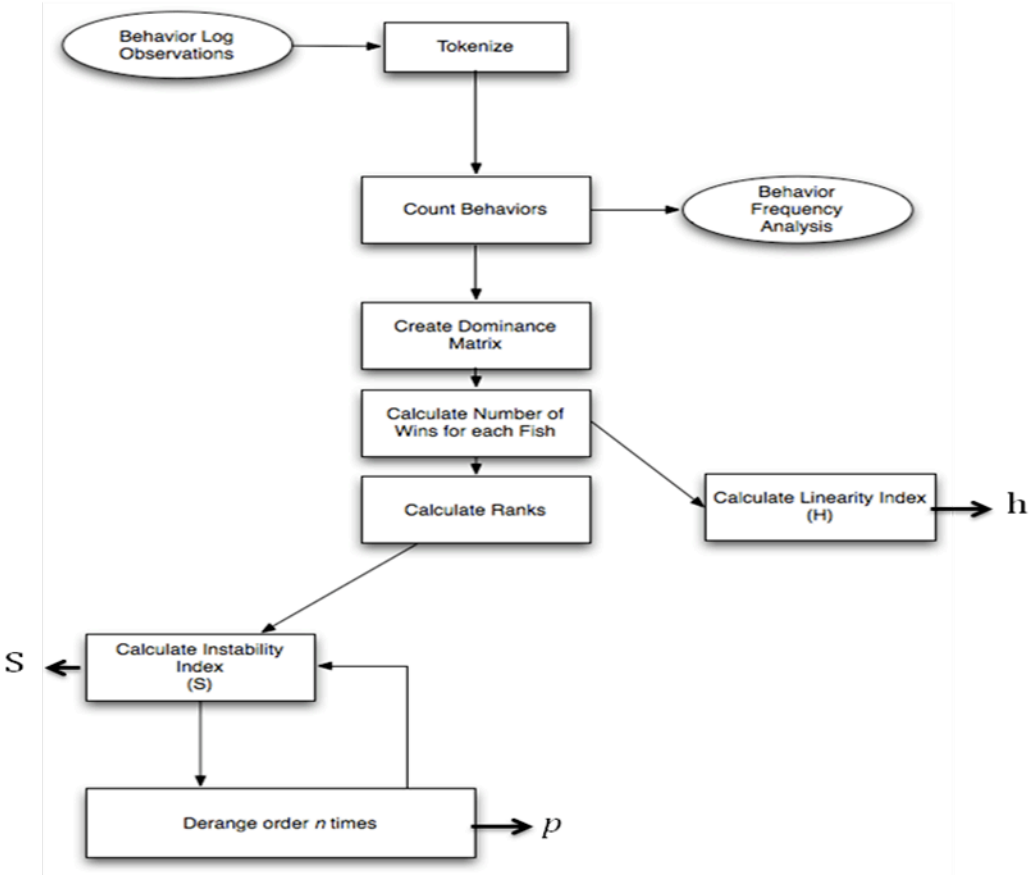
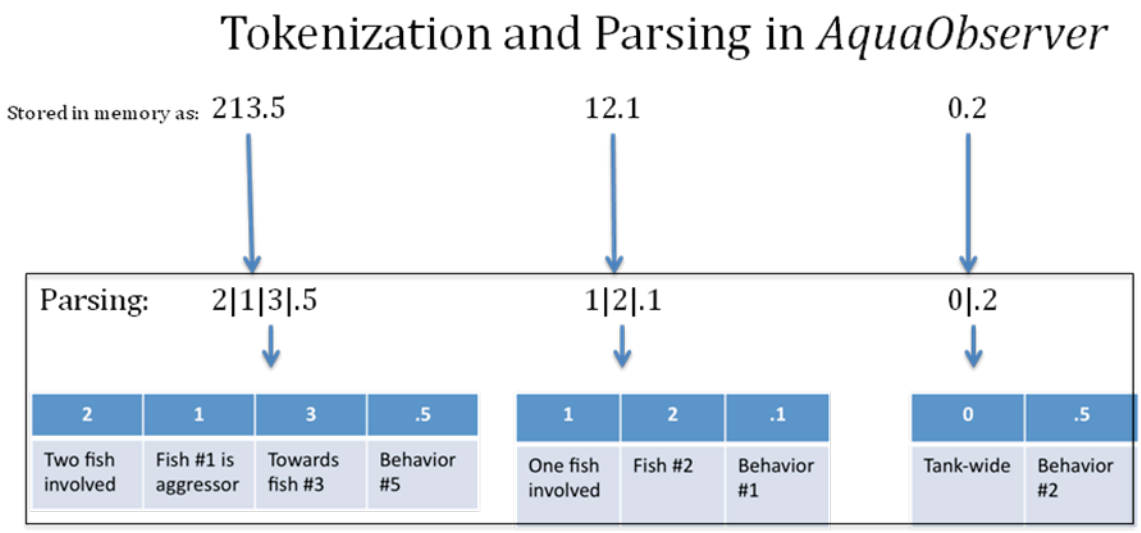


Figure 8. Information flow in AquaObserver's BLMAn. This diagram offers a simplified version of the way in which behavioral data is obtained and analyzed. Outputed sociometrics and statistics have been highlighted to show at which stage of analysis they are calculated

.

Figure 8 shows the flow of information in *AquaObserver* Behavior Log Manager and Analysis module (BLMAn). The user creates the Behavior Log file using the Observation Logger and Video File Viewer, and the annotations are saved in the log file. After the user specifies the type of analysis and the files to be analyzed, all behavior log files pertaining to an experiment are tokenized, *in memory.* Tokenization is the process of assigning any behavior a token value that represents it in a reference table, termed the tokenization table. *AquaObserver* generates a tokenization table based on all log files in an experiment. The tokenized data can be parsed and analyzed for behavior frequency in

a straight forward manner. Tokenized data encodes information about the direction of the aggressions, and the dominance matrix can be constructed from it (**Figure 9**). The tokenization table is displayed before final analysis.



Behavior Code	Behavior
1	CC (color change)
2	Fed (Fish presented with food)
3	Chase
4	Tank is being filled
5	OM (nip)

Figure 9. Tokenization of behavior in *AquaObserver* allows the program to quantify, sort, and categorize behavior by interpreting the tokeinized data.

Analysis of Frequency and Behavior Sequences

Behaviors can be analyzed for frequency, transition between behaviors (often termed sequential analysis), and circadian rhythms. These tools can be applied to build ethograms at both group and individual level, or to analyze behavior patterns of animals. Furthermore, simple statistics can be quickly obtained to assess the significance of such patterns.

To analyze circadian behavior, each behavior log file is given a time index, which corresponds to the time the recording took place. The computer automatically aligns the relative time signature in each file, with this index.

Transitions between two behavioral states, or between two different behaviors, can be treated and modeled as Markov Chains. Markov chains are classified according to their order. The order of the chain is equivalent to the number of "links" that one will have to travel back in the chain to predict the chain sequence. In a true second-order Markov Chain, the first event in the chain and its following event (second), can be used to predict the identity of the third event in the chain. Similarly, in a first order Markov Chain, the event or state determines the probability of the identity of the next event in the chain. Zero-order chains are ones in which the events do not have discreet transition probabilities. Hence, in zero-order Markov Chains, the behaviors occur in a random order.

I have been able to identify at least one such "group" chains in the Gilthead Seabream, in which the action of one individual triggers a cascade of reactions from one or more other individuals in the group (**Chapter 3**). To analyze the order and significance of these behavior chains, *AquaObserver* computes a G-test statistic and a χ^2 statistic for each transition. The G-test is a more generalized form of the χ^2 test, and is based on the same distribution as the χ^2 test. The G-test is especially helpful when the difference between the expected and observed values is large, which often happens with infrequent behaviors.

Ranking Algorithm

In assigning ranks to each animal based on the observations of the embedded dyads, one of the difficulties is that each win or lose event observed does not represent an independent observation (e.g. because of winner or looser effects). To circumvent this problem, de Vries (1998) suggested that the dyad and not the dominance event should be considered the observational unit. Given a pair of fish, fish A and fish B, embedded in a social group, this model has 4 possible outcomes: 1. Fish A wins; 2. Fish A looses; 3. Fish A ties with Fish B (both have equal number of aggressive acts); and 4. Fish A does not engage Fish B. The later is what de Vries (1998) termed an "unknown relationship".

After creating a basic dominance matrix, the software analyzes all possible permutations of dyadic encounters that can occur among a group of animals consisting of *n* number of individuals to create the win-loose dyad matrix. For each dyad a threshold for ties is set for the minimum difference between aggressions by Fish A vs. Fish B that will be counted as an absolute win. For each fish win, the winner is given 1 point, while the looser is given 0 points. For each tie, (when threshold is not exceeded) each fish in the dyad is given 0.5 points. Selecting a low threshold or using 0 as the value for threshold yields the most stable hierarchy.

If the pair never engaged in any aggressive behavior in the observation period analyzed, the results from the last known encounter are applied to calculate the wins and assign the points. I termed this feature the *stack*. Usually the rule is to declare such a relationship as "unknown" or "unresolved". However, I assume: 1. A perfect observer; 2. A stable relationship between observation periods; and 3. Animals do not always interact once they have established a dominant-submissive relationship. When assumptions 1 and

2 are met, the last observed relationship is true and has been maintained since the last observation period. When assumption 3 is true, the only way to know the dominant-submissive relationship between two animals that have a stable relationship is by analyzing the past encounters, because it will take many observations to see an interaction between them (since now they have a stable relationship). Thus, we fulfilled this assumption by observing the animals up to 7 times per day for 30 minutes, for a total observation period of 210 minutes/day (23% - 50% of the total daylight activity period, depending on the day of the year).

If unknown relationships occur, 0 points are given to each member of the pair in the relationship until an interaction is observed and a winner can be assigned. Thus, this algorithm minimizes the occurrence of unknown relationships, but such relationships can still occur, especially when analyzing the first observation logs or when observational data is not grouped on a per-day basis. The latter case can potentially violate the assumption of a perfect observer and render the algorithm invalid. Thus, I grouped all 6 or 7 daily observations into one day and reported and analyzed rank data accordingly.

Each fish is ranked depending on the number of win points that are accumulated from the algorithm described above. Rank ties are handled in a manner similar to the algorithm used in the Wilcoxon Signed-Rank Test (Rosner, 1995). Thus, a fish may have a rank with a decimal value and more than one fish may have the same rank. Note that this type of tie is not the same as a tie among dyad encounters, which is known as a dominance tie. A rank tie implies that both animals have won the same number of encounters, whereas a dominance tie implies that two animals have had the same number of dominant-aggressive interactions in opposite directions. Rank ties are very common

when the hierarchy is not linear, because they are often the result of circular elements in the group, while dominance ties may be the result of dominance relationships that have not been decided yet or they may also result from a lack of linearity.

Our algorithm for determining rank is analogous to determining the number of fish that are subordinate to each fish in the group. This latter technique is known as the Netto Method and was the algorithm implemented in the first version of the now widely popular sociometric/ethological software MatMan (DeVries et al., 1993). The Netto Method has been shown to be highly effective in finding the true dominance order of a group of animals when compared to other, more complex algorithms (Hemelrijk et al., 2005). It is also very similar to the procedures described by Langbein and Puppe (2004) in their review of methods for analyzing dominance at the dyad level. Thus, with the exception of the manner in which unknown relationships are handled, *AquaObserver*'s ranking algorithm is similar to accepted and published ranking procedures.

Linearity and other Sociometrics

Dominance hierarchies in animals can organize in several ways (Wilson, 1973). Some of the elementary and emergent properties can be measured using published behavior metrics (Review: Langbein and Puppe, 2004). For example, the linearity of a dominance hierarchy can be measured using the Landaus linearity index *h* (Landau, 1951). The number of circular elements can be measured using Kendells Coefficient *k* (Kendell, 1962). Under most conditions, Kendell's and Landau's index have similar values as both measure group-level (emergent) properties, which arise from dyadic interactions. The Directional Consistency Index (DCI) can measure the strength and

direction of the dyadic relationships (van Hooff and Wensing, 1987 in Cote, 2000). *AquaObserver* can calculate and report any of these parameters.

AquaObserver also reports the number of unique ranks that the algorithm finds in each of the observation periods. I call this parameter, the number of levels in the hierarchy (NLH). If NLH equals the number of animals in the group (N), the hierarchy is perfectly linear (h=1). However, the converse is not true: a Landau's index of 1 can still be calculated for a hierarchy in which NLH does not equal N.

Instability Index

The instability of a social group will be reported based on changes in ranks between observation periods, using the following index that I developed:

$$\mathbf{S} = \frac{\sum_{i=1}^{n} (\Delta Rank_i)^2}{M_n}$$

$$M_n = \sum_{i=1}^{n} (2i - n - 1)^2$$

Where, $\Delta\mathbf{Rank_i}$ is the difference between the rank of the ith animal in one observation period and its rank in the next observation period and n is the number of animals in the group. $\boldsymbol{M_n}$ is the Maximum value of $\sum_{i=1}^{n} (\Delta Rank_i)^2$ for a given n. In tetrads, where n=4, the value of $\boldsymbol{M_n}$ =20. The $\boldsymbol{M_n}$ value can only be achieved when the hierarchy is completely inverted. Thus, when S=1, the hierarchy is inverted and the instability has reached its peak. Conversely, when S=0 no animals have changed rank,

and the instability is low. Indeed, the index can be defined as a value that approaches 1 as the rank changes increase or approaches 0 as the group stability increases.

Squaring the value, as opposed to using the absolute function to eliminate any negative signs, allows the index to be less sensitive to small changes in ranks and more responsive to large rank changes. This is especially important when one considers the effect of rank ties on stability. For example, when two fish share the same rank in the hierarchy (i.e. Fish A: 1.5, Fish B: 1.5), but the animals are "un-tied" in the next observation period (i.e. Fish A: 2, Fish B: 1), a change in the rank will occur between the periods. Constant changes in ranks due to these tie/untie events will occur when the animals involved are equally dominant (equidominance) and at the same "level" in the hierarchy. I did not want the instability index to be affected drastically due to the presence of equidominant animals. Instead, I conceived the index as a way of assessing the stability of the dominance structure of a group of animals as a whole. By squaring the magnitude of the difference in rank between observation periods, small rank changes (0 to 1) create only small changes in the value of the index, while large rank changes (>1.5) create even larger changes in the value of the index.

Groups with strong linear hierarchies should also have stable hierarchies. Although Landau's index does not take instability into consideration, the behavioral and biological mechanism that underlie the formation of dominance groups imply that individuals in linear hierarchies do not change the dominance/submissive relationships that exist among themselves, because doing so would introduce non-linear elements into the group. Thus, instability and linearity are indirectly related.

Based on this idea, we can predict that Landaus index H should be inversely correlated to the S index. Because both measurements use the same rank data but are based on different

Landau's *h*	S_{AVG}	S_{MEDIAN}	S_{RANGE}	N
1	0.11	0.1	0.3	11
0.2	0.28	0.23	0.73	10

Table 1. The relationship between Landau's index *h* and the stability index *S*. The "perfectly" linear hiearachy had more stability. The hierarchy with no linearity, was more unstable and showed more variation in calculated stability, perhaps due to random rank assignments.

calculations, we can test if this prediction is true. Calculating both indexes for a set of observations of 4 seabream tetrads (Table 1), shows that when the hierarchy was perfectly linear, the stability was usually high. When the hierarchy was not linear (e.g. egalitarian), the stability was low or high. Thus, a linear hierarchy is usually stable, but the converse is not always true, because ranks may be assigned randomly by the algorithm when the hierarchy is egalitarian or has too many circular elements.

Permutation test for the Instability Index

Like Landau's index, the instability index is a dimensionless quantity. Because of this, it is difficult to evaluate the significance of values between 0 and 1. To provide a measure of significance for the average index for all observation periods, I designed a randomization procedure in which the order of the observations is randomly changed before calculating the average index, and the process is repeated many times. Recall that the order of the observation affects the index, because the index depends on rank changes that occur between two observation periods. By randomizing the order, the significance of the average instability index can be assessed. A permutation test belongs to the family

of exact tests. It is a useful statistical tool when other parametric or non-parametric tools can't be applied because the underlying distribution of the sample is unknown (Anderson, 2001). The test requires shuffling the original order of the observed fish ranks, calculating the average for this set, and then comparing this calculated average to the observed average of the original set. The process is repeated again until a two-tailed test *p*-value can be calculated based on the number of times that a randomly ordered set of fish ranks has a value different than the observed average S. The significance level for the permutation test can be set at α=0.05. Thus, the goal of the permutation test is to find if 5% or less of all the generated randomly order sets have an average stability that is not equal to the observed average stability.

Generating Derangements of the Original Ordered Set of Observations

The first step in this randomization procedure is to shuffle the original ordered set several times to obtain a number of "derangements" or randomly arranged sets. A problem immediately occurred when I first tried to apply this algorithm using Microsoft's VB 6 random number generator in the first implementation of this algorithm: the VB6 random number generator does not have a large *period* and it was unable to produce the number of unique permutations needed. The problem was resolved by using a Mersenne Twister pseudo-random number generator in the algorithm. The Mersenne Twister algorithm that I integrated into *AquaObserver* was obtained from a source code I found on the Internet, and which was licensed on *public domain*. Mersenne Twisters have extremely large *periods* and can generate lists of numbers that are *equidistributed* (Matsumoto and Nishimura, 1998). For these two reasons, the Mersenne Twister is especially suited for statistical procedures, such as re-sampling, exact tests and Monte

Carlo simulations. This is because the algorithm can generate a long (*large period*) list of unique (*equidistributed*) numbers. In the case of the permutation test, an exact test, the algorithm is used to generate a unique number of derangements.

With each one of the generated sets of derangements, the algorithm calculates a new instability index (S_r) and then it repeats this process for each derangement generated. The number of derangements that can be produced from a group of observations depends on the number of observations. In combinatorial mathematics, a derangement is a permutation of the set in which none of the elements in the set are in the original order. Normally, this is given by the *subfactorial of n*, also expressed as $!n$, which by definition is a number smaller than the factorial of n or $n!$.

However two unique constraints apply to the S index permutation test: 1) We should not care if the observations are in the same position or different positions in the list, as long as they are surrounded by observations that are not in the same position. 2) Because the calculations involved the squaring of the difference between observation periods, the calculated S will be equal for the original ordered set and the reverse ordered set. Thus, the total number of possible unique combinations that can potentially change the calculated value of S is therefore, $n! - 2$.

As the number of observations (or group of observations), becomes larger, the value will approximate the factorial $n!$. For an experiment that ran for 5 days in which the daily stability is to be calculated, the total number of permutations is:

$5! - 2 = 118$

Thus, if the index was calculated on a daily basis for an experiment that lasted 5 days, then there are 118 ways in which the observations can be ordered and 118 possible values of S that could be different from the observed average S (S_o). If a randomization algorithm is run to generate most of the 118 possible ordered sets, the probability distribution for S can be estimated. Note that if a period of 10 days is considered, then the number of times that this procedure needs to be repeated is approximately 10**!,** or **3,628,800**. In such cases, most statisticians suggest to sample a large number of the derangements (i.e. 5,000) to calculate the p-value.

Chapter 1B: AquaObserver, as a tool for the measurement of fast physiological color changes in aquacultured fish housed in heterogeneous lighting environments.

Introduction

In fish, communicating dominance status might be important. In the previous section, I discussed how *AquaObserver* can be used to quantify fish rank, group rank stability, and linearity, which all depend on aggressive behaviors that are easily and readily observed. Such behaviors convey information about the dominance status of the individual to the fish that receive the behavior, those fish that observe it, and the human observer, and are thus the most widely used indicators of dominance rank. However, posturing is another form in which animals might relay dominance status information to others. Such postures may consist of elaborate aggressive displays, or simple and fast whole-body or pattern-specific color changes. Indeed, such signals may play a role in the formation and maintenance of dominance hierarchies.

Teleost fish have groups of neuronal-derived pigmented cells embedded in the epidermal tissue which allow the animals to change the pigmentation intensity of their entire body or express a different pattern of body coloration (Review: Fuji, 2000). The most common type of pigmented cells in fish is the melanocytes. These cells are directly innervated by the sympathetic nervous system. Thus, both plasma hormones and symapthetic activity affect melanocyte activity. In response to specific stimuli, melanocytes disperse or aggregate their pigment granules, also known as melanophores. Dispersion of melanophores leads to body darkening. Conversely, aggregation of the pigment granules leads to skin lightening. Both processes involve activation of the cell transport machinery (Logan et al., 2006). The quick aggregation or dispersion of

melanophores, known as a physiologic color change, causes transient color changes in fish.

Several hormones are involved in color changes (Review: Sugimoto, 2002). Alpha-Melanocyte Stimulating Hormone (α-MSH) is secreted by the hypothalamus, and stimulates dispersion of melanocytes. Similarly, Melanocyte Concentrating Hormone from the hypothalamus stimulates concentration of melanophores, which leads to whitening of the skin (Kawauchi and Baker, 2006). Conversely, stimulation of melanophores leads to darkening. Increased sympathetic activity, such as observed during a time of stress or a "fight or flight" response, leads to concentration of the melanophores (Review: Fuji, 2000). Both MCH and norepinephrine, have similar effects on melanophores. MCH, and NE initiate apoptotic pathways on the melanophore and also decrease their dendricity. α-MSH is anti-apoptotic and promotes growth of the melanophore. Thus, depending on the duration of the stimuli, a color change can be permanent or transient. Transient color changes are known as physiologic color changes. If there are changes in the structure or number of melanophores, it is known as a morphological color change.

In artic char, fish pigmentation intensity is correlated with the dominance status of the animal (Hoglund, 2000). Tank color may affect fish color through the background adaptation response (Doolan et al., 2008). In some species, white raceways increases serum cortisol levels (Rottlant atl al., 2003). Tank color can also affect other physiologic parameters, such as hunger (Amiya et al., 2008).

Because physiological color changes are difficult to measure, especially within the social context that they normally occur, most research in this field has focused on

morphological color changes. For example, Hoglund et al. (2000) was able to correlate fish color to dominance rank because social stress causes morphological color changes in artic char. Thus, the authors could measure the color of the fish by taking pictures of each individual before and after the experiments.

In this paper, we present a software-based image analysis approach which allows the measurement and quantification of physiological color changes that occur in the context of aggressive behavior in fish.

Design Considerations and Program Features

The increased efficiency of compilers and exponentially increasing computer speeds, allow high-level languages (e.g. Visual Basic) to efficiently handle most low-level, computationally intensive applications such as image analysis and computer vision. Thus, I was able to successfully code this component in Visual Basic. *AquaObserver*'s image analysis module consists of several classes that encapsulate all the necessary functions and subroutines. It uses a public domain library of classes, courtesy of VBAccelerator.com, that allow an image from an **AVI** video file or a **BMP** to be transferred into a VB array.

All data generated by *AquaObserver* is outputed to comma-separated value files (**.CSV**), which can be read by Microsoft Excel or other commercially-available applications. Using the behavior log's time-stamped files, color changing data can be correlated to aggressive behavior. This can be accomplished in several ways, including measuring the animal's color at each aggressive event or by correlating the time indexes of color changing events and aggressive behaviors.

Computer Vision

Computer vision technology has been around for many years. A combination of specialized hardware and software allows computers to perform tasks that would normally require human intervention, which is why computer vision is often considered a sub-discipline of Artificial Intelligence. It involves a multi-disciplinary combination of both applied and theoretical science. Computer vision borrows techniques from theories and contributions from various fields of biology, mathematics and engineering.

The first step in computer vision is image acquisition. Usually, one or more cameras provide the necessary information that the computer system will process to complete the assigned task. After acquiring the image, additional steps are taken to process the image information even further, until the relevant features can be extracted:

1) **Pre-processing**: Images may have electronic/pixel "noise". The first step might be to remove this noise. Also, the image might need to be color-corrected, or require enhancement of contrast or illumination.

2) **Segmentation**: Where are the regions of interest in the image? Segmentation is the process of finding these regions. For example, if the software needs to find vegetation in a video of a desert, it might segment the image by color: all green pixels are found and assumed to correspond to vegetation.

3) **Object Identification**: Positively identifies objects as objects of interest, assigns numbers, counts them, etc.

4) **Feature Extraction**: This step might come before or after identification of objects, depending on the specific task. In this step the computer will attempt to find edges, contours, corners, etc. Shape information might help identify objects

or even help segment the image, but usually it is only relevant for higher level analysis.

5) **Pattern Analysis/Feature Analysis/Tracking**: Areas of the object detected can be analyzed to detect the object movement axis, track the object and predict its next position, and the pattern or texture of the object can be assessed based on a series of complex computations.

6) **Higher level processing (Artificial Intelligence Engine, Classifier)**: The computer might be tasked to make decisions of which objects to analyze further depending on the features extracted. Often at this step, a machine-learning component can be added or, in the case of *AquaObserver*, a *classifier* (a program that can categorize data based on specific criteria) can accept or reject objects as being a fish, a shadow, etc. based on the extracted features.

Many standard techniques for each of these steps have been developed to deal with most real-world applications of computer vision. Still, specific applications need different approaches in each of these steps, and programmers may elect to omit steps, mix approaches and techniques, and/or perform them out of the order described above.

The field of computer vision is continuously evolving to take advantage of increasing computing power. Thus, programmers and mathematicians consistently publish real-world and theoretical solutions to general problems (e.g. Mittal et al., 2009) or develop optimized techniques for a specific subset of problems (e.g. Stien et al., 2007). Here, I contribute to both the field of applied computer vision, by developing a set of tools that attempt to solve a very specific problem, and also to a minor degree, to the field of machine vision, by simplifying the existing technique of segmentation by color-ratios.

In this section, I will detail the approach that I took with *AquaObserver* and I will explicitly underline the major contributions of my work in these two fields.

Like the costly, widely popular, and commercially-available, *EthoVision* from Noldus Information Technologies, *AquaObserver*'s Fish Tracking and Color Analysis module is a state-of-the-art computer vision system. However, *AquaObserver* has many advantages over this system. For example, the program was designed exclusively to be used in aquaculture systems, and it was developed to detect and identify fish in an environment that most programs, including *EthoVision*, would find very challenging. This is because *AquaObserver* is a custom program designed to work with the specific constraints and peculiarities of my experimental setup.

Application Design

The entire application consists of 3 separate "modules" or task-specific components: a behavior logger and video player, a behavior analysis, and the tracking/color measurement/analysis component that I will now describe in detail. Editing and debugging the application is simplified by the principles of Object Oriented Programming. A simplified diagram of the computer vision module is shown in **Figure 10**. However, the entire application consists of 10 Classes, almost 40 "forms" or windows (each window's layout is usually designed by the programmer and is called a "form"), and more than 150 pages of code, with most of it devoted to the computer vision component. The program's complexity stems from the inherent complexity involved in solving computer vision problems and not out of sloppiness in its algorithms or implementation. The program speed is limited by disk access, as it retrieves each frame from the video file, and is not a result of over-reliance on *naive* algorithms: many of the

program's core algorithms have been optimized. Most of the image analysis and computer vision algorithms are contained in one Class, **ImageProcessor**, and the tracking and track analysis components reside in another Class, **TrackMan**. This simple two-Class approach simplified programming conceptually but required more effort to maintain and debug the code. Nevertheless, *AquaObserver* is a powerful application that has the potential to be applied to other fields, such as quality-control in aquaculture, fish sorting, ecology, and tracking of other species of animals.

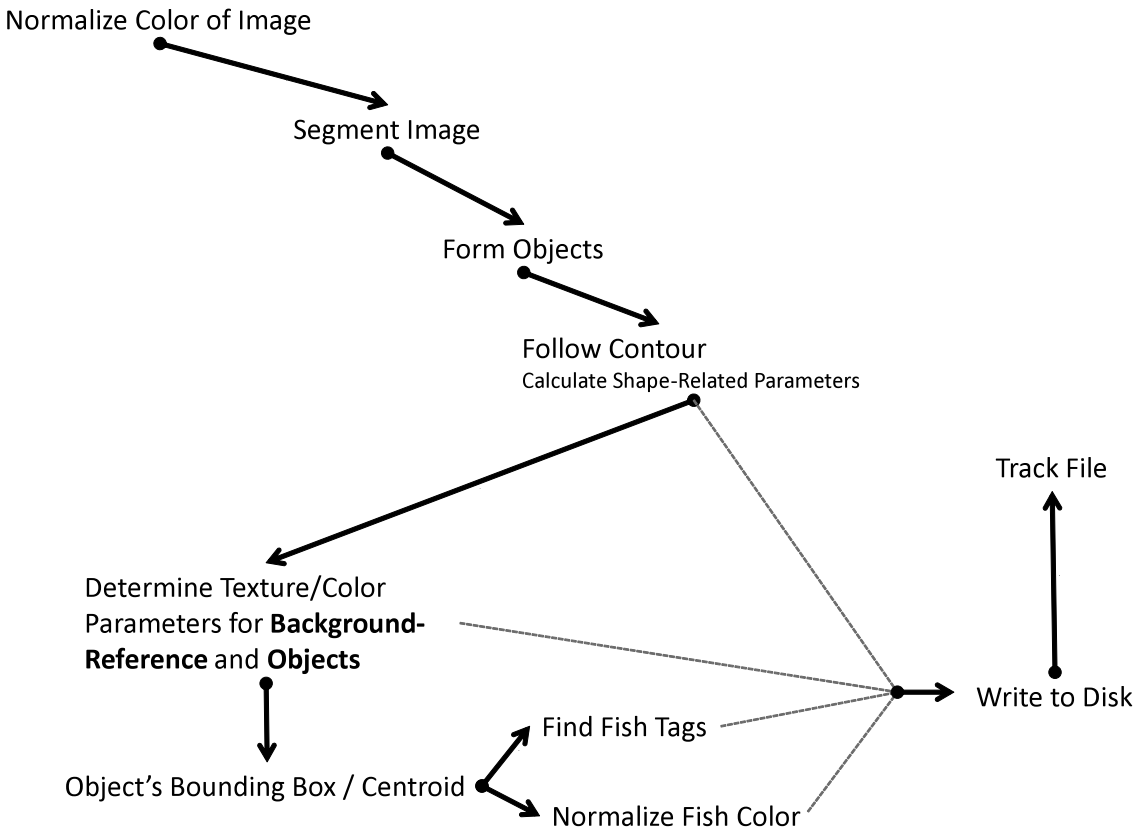


Figure 10. Simplified flow-diagram that shows the different steps of processing of images in AquaObserver.

Digital Filters

Standard brightness and contrast filters can be applied to each frame in the video file. Also the color normalization filter (discussed below) can be used to change the color tint of the videos. Also, two filters are provided that can be used to enhance object detection under uneven illumination. A logarithmic brightness filter (LBF), applies a user-defined exponential function to each pixel to enhance its intensity. LBF increases brightness for areas that are not well illuminated, while areas under high levels of illumination should not change or show little change.

The second, and perhaps most useful, filter for unevenly illuminated images can "mask" areas of the background that are not well illuminated (i.e. one corner of the tank) and only increase brightness to those areas. This filter is analogous to using layers in Adobe Photoshop to correct for backlit subjects. Indeed, it acts as a digital "fill-in" flash.

Fluctuations in water quality and Background Changes due to Biological Activity

A common problem in aquaculture tanks is the accumulation of organic pigments that give the tank water a yellowish or brownish tint. Although the systems are designed to export or filter such compounds using foam fractionators and systematic water changes, yellowing of the water can't be completely avoided. Similarly, the high biological activity of these systems leads to the accumulation of "biofilm", often from bacteria or algae present in the system. The brown or green biofilm can be found in the walls and bottom of the tank, as well as other exposed surfaces under the water such as pipes and filters. The presence of this film presents two problems. First, the wall and bottom of the raceway can become partially, or totally, green or brown in color. This leads to technical difficulties when using some of the standard and common image

analysis techniques. Second, because fish scales are highly reflective, the presence of an increased amount of biofilm in the tank walls may lead to a change in the measured color of the animal.

Increased accumulation of algae and bacteria on the walls and sides of the raceway is part of the larger problem of tracking animals in a biological arena. Uneaten food particles, air stones or probes that shift position as the animals change swimming speed or direction, etc. are sources of changes in the image. However, many published algorithms assume the background arena does not change, which poses a problem.

The process of finding objects of interest in an image is called *image segmentation* (Parker, 1996). The most commonly implemented segmentation algorithm used by animal behavior software is background subtraction. In this algorithm, a reference image of the arena with no animals (background reference) is compared to the image to be segmented. Thus, the program can find the areas of the image that have changed compared to that background reference.

The reference image is often created by taking a picture of the arena with no animals. However, as discussed above, such technique will not be adequate for aquaculture systems, because of visible daily changes in the raceway. An example of this problem is shown in **Figure 11a**, a screenshot of a video taken 24 hours after **Figure 11b**.

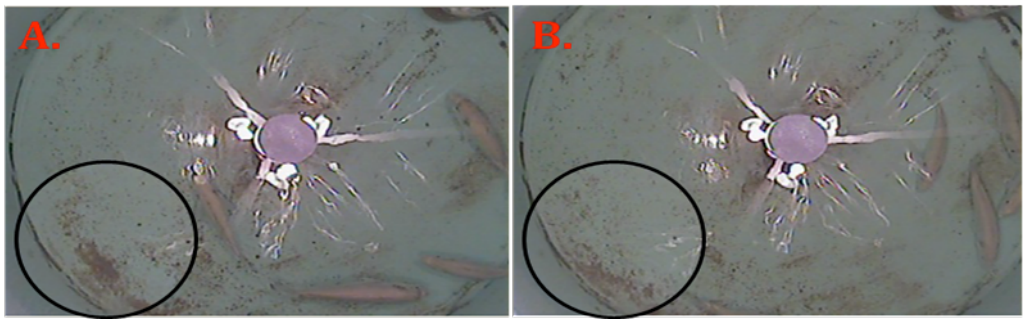


Figure 11. *The effect of 24hr of biological activity on the image "background" or raceway. (A) was taken exactly 24 H after (B). The area highlighted by the circle marks a region that may show problems if segmenting using a more standard, "no-animals-in-arena" background reference.*

To circumvent this problem, *AquaObserver* allows each videofile analyzed to have its own background reference. Two software solutions are available. The user can create it by "stitching" together several frames using *AquaObserve* (**Figure 12)** or alternatively, by using *AquaObserver* "autoreference" feature, which creates a background image by averaging a large number of frames from the video file. Neither of these solutions is completely innovative, as similar algorithms have been previously developed, although perhaps these solutions may have never been applied to the measurement of fish color in the context of behavior observation. Both solutions work well with aquaculture raceways.

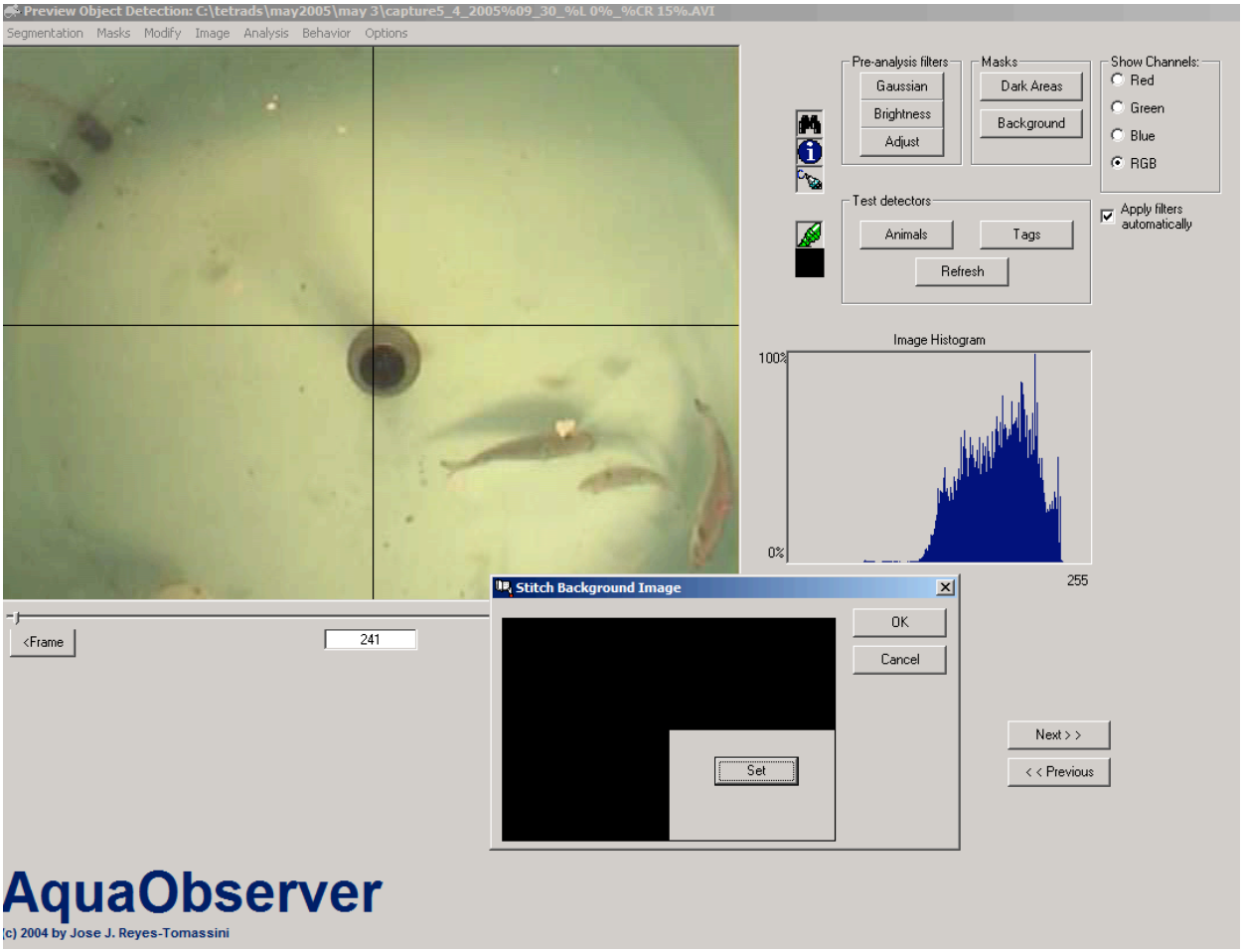


Figure 12. *A reference image can be created by using sections of different frames. In the image above, three segments have been taken from this frame to create the background reference. Only one more segment is needed to create the final reference image.*

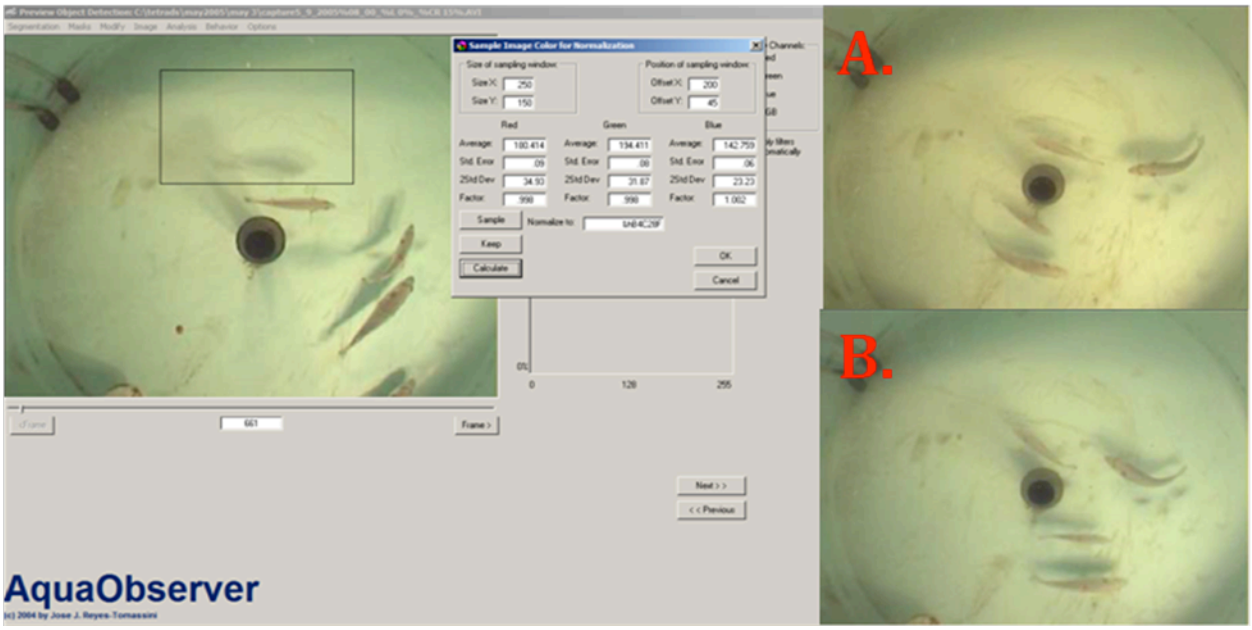


Figure 13. Color normalization is a two-step process. First, the image to be used as reference is sampled at a randomly chosen area. To normalize all frames in a video file, the same process is repeated using any frames where no animals are present in the sampled area. The computer then compares the color of the sampled section in the reference image to the color of the sampled section in this frame. It establishes a normalization factor to be applied to every pixel in every frame of the video file. In insets (A) frame before, and (B) after normalization. The frame in the main figure was used as the reference. Notice the change in overall image color from yellow-green to blue-green.

AquaObserver can also normalize each image in the video file to compensate for shifts in tank or water color. This is accomplished by selecting a small portion of the tank background as a reference in one file, then using this reference values to normalize all video files analyzed subsequently. The pixel values in the image can be normalized so that all subsequent color measurements obtained from the images are correct and values obtained for a given color remain consistent across different video files (Villafuerte and Negro, 1998). **Figure 13** illustrates the process of color normalization using *AquaObserver*. The original image is yellowed, probably because of water quality problems. After normalization, the image has a blue-green tint, which is exactly the tint

of the original image against which it was normalized. As with the previous solution described above, normalization is not innovative *per se.* However, it has never been applied in this way. The normalization step is important because it allows comparing fish color across different experimental groups and different filming days. To my knowledge, this is the first time such an algorithm has been used to study fish color-changing behavior in a long-term observation setup.

A digital image can be defined as a mathematical function *f(x,y)* that maps the brightness values of each pixel in the image. Color in this type of image is defined within a color-space. In the RGB (Red-Green-Blue) colorspace each pixel is represented by three brightness values (aka channels): Red, Green and Blue. For convenience, we will define a channel selector *s* for the image function *f(x,y)* in all the following algorithms, where s=1 for Red, s=2 for Green, and s=3 for Blue.

The algorithm that normalizes the color of all the frames in a video file is extremely simple. Note that here $\bar{b}$ is the channel average intensity, and *C* is the channel correction factor applied to each pixel in the image.

Given an *n x n* rectangular section of a reference image and a no-fish background image where the same *n x n* region can be sampled, then for each channel *s:*

$$\bar{b}_{reference} = \frac{\sum_{x}^{n}\sum_{y}^{n} f(x,y)}{n}$$

$$\bar{b}_{background} = \frac{\sum_{x}^{n}\sum_{y}^{n} f(x,y)}{n}$$

$$C = \frac{\bar{b}_{reference}}{\bar{b}_{background}}$$

$$f_{normalized}(x,y) = f(x,y) \times C$$

Repeat for all channels.

Image Segmentation Algorithm

Subtraction of the background reference from the frame of interests allows the program to find any areas where the image has changed. It is a straight-forward procedure that can be implemented by the most *naive* algorithm and still yields good results. A threshold value (T_s) is used to define the maximum allowable difference between the two pixels before a pixel is considered to not be a part of the background.

If the image to be segmented is defined as a function $F_{frame}(x,y)$, a no-fish reference called $F_{reference}(x,y)$, *s* is the RGB channel selector, and g(x,y) is a segmentation mask containing a bit-wise mask that represents the identity of each pixel in the image (segmented or not segmented) then:

For the <u>blue</u> channel image extracted from frame *n*:

$$\mathbf{f_0(x,y) - f_n(x,y) = d}$$

$$\mathbf{g(x,y)=}\begin{cases} 1, d \geq T_s \\ 0, d < T_s \end{cases}$$

Note that if $d < T_s$ the pixel is considered to be a background pixel, but when $d \geq T_s$, the pixel is considered an object pixel.

Segmentation by illumination-invariant color ratios

Because the lights are positioned on the side of the tank in some of my setups and because the usual raceway lights are not very bright and are usually setup as a one point-source light, I have encountered a persistent problem with shadows. This problem halted my original progress with the program, because it created an arena full of shadows. The

program had difficulty discerning shadows from fish, and often detected the conjoined figure formed by the shadow and fish as a single entity.

Almost all traditional image segmentation algorithms will have difficulty under this situation. A shadow represents a change in the background. Setting the detection threshold to a very high value does not work well, even if the full color information is used. This is because seabream are dark fish, and the color changes that I wanted to study often cause the animal to become very dark or turn into a very light grey color. Because fish swim at different levels in the water column and because the arena is circular, often a shadow was cast on a fish which made detection nearly impossible. This also negated the use of a classifier to systematically label and ignore shadows, something that I attempted once I stumble upon this road block and which led to a highly sophisticated computer vision system, which I will discuss later. It is also important to note that other commercial solutions, such as *EthoVision*, would most likely be confused by shadows, even when they use background averaging or subtraction: each fish will cast its own shadow in a different way and the effects of two shadows overlapping, a common occurrence when two fish are swimming close to each other, would be even more problematic to avoid (convergent shadows are very dark). A different approach is needed.

Illuminant-invariant color angles and color ratios are now commonly used in many robot vision applications and in the field of computer vision (Finlayson et al., 1996*;* e.g. Tiwari and Gallager, 2003; Wanderley and Fisher, 2001). Because it is not as sensitive to heterogeneous illumination as other approaches, the implementation of this algorithm can enhance the segmentation of the image, even when shadows are present.

The angle or ratio between the three components colors of the RGB colorspace describe each of the unique colors represented in the colorspace, regardless of its intensity. Thus, the ratio between the three of them is insensitive to changes in illumination intensity.

I implemented a *naive* version of the algorithm. This first version was very hard to adjust, since it had a low and high threshold for each color (i.e. 6 parameters to adjust its sensitivity), multiple comparisons, calculations, etc. It was not simple to adjust to the different lighting conditions of the different experimental arenas I filmed. These problems led me to create an entirely new algorithm based on Finlayson et al. (1996) idea, but using a relatively simple formula that had only two parameters. I named this algorithm the Background and Shadow (BAS) filter. It is thoroughly explained and derived in Appendix A. The following is a simplified explanation of the algorithm.

After subtracting the pixels, *AquaObserver* compares the RED: GREEN: BLUE ratio in the background reference image to the RED: GREEN: BLUE ratio in the frame to be segmented. It then calculates the ratio of that ratio. This ratio of ratios is compared against a threshold value that indicates how similar the background (no-fish) pixel color is to the pixel being segmented. Thus, it is the analogous of the background subtraction but using the more stable color ratios as a basis for comparison. **Figure 14** illustrates the effects of the BAS filter on the segmentation of an image that has shadows. The raw image is shown in **A**, and the effect of a simple background subtraction is shown in **B**. Applying the BaS technique gives a good relatively good, although not perfect, detection of all the animals. The alternative, which is to use background subtraction with an increased threshold of detection, precludes detection of the smaller fish in the image (**D**).

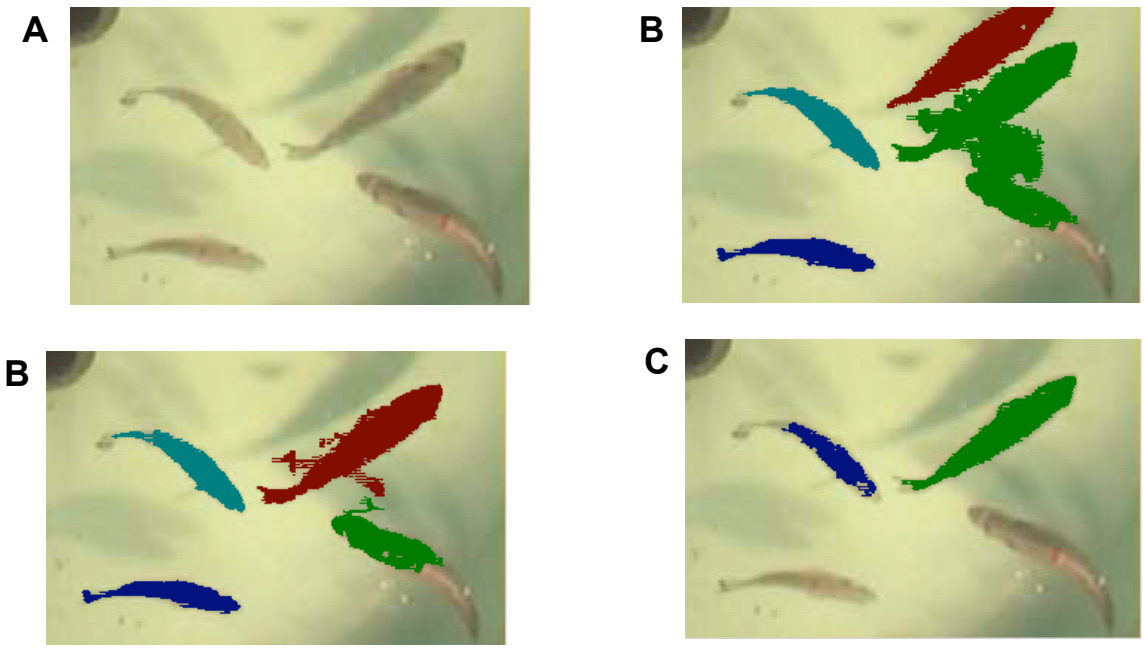


Figure 14. (A) Image to be processed. (B) With the subtraction method and a low threshold. (C) With same threshold and BAS filtering. (D) Higher threshold and no BAS filtering. Notice that some of the animals are not segmented from the image.

To apply the BAS filter, a ratio *r* is first calculated:

$$R_{BAS} = \frac{\left(Blue_{frame}(x,y)\right)^{\beta} \times Green_{reference}(x,y) \times \mathrm{Re}\, d_{reference}(x,y)}{\left(Blue_{reference}(x,y)\right)^{\beta} \times Green_{frame}(x,y) \times \mathrm{Re}\, d_{frame}(x,y)}$$

When $R_{BAS} >> 1$ or $R_{BAS} << 1$ then the pixel evaluated is considered to be an object pixel. Two thresholds are required, namely T_L (low bound) and T_H (high bound), so that if $T_L > R_{BAS} > T_H$, then the pixel is too different from the background reference and we can safely consider it an "object" pixel to be segmented. The formula weights the intensity of the blue channel more than that of the other channels. Its derivation is shown in Apendix A.

Both the subtraction and BAS filters are implemented in tandem, so that the criteria for segmenting a pixel into the new segmented image g(x,y) becomes the logical

"AND"ing of the output of these two segmentation algorithms. In mathematical terms *g(x,y)*, which is the bit-wise mask containing the segmented pixels, becomes:

$$\mathbf{g(x,y)}=\left[\left(\mathbf{g(x,y)}\begin{cases}1, d \geq T_s \\ 0, d < T_s\end{cases}\right) \cap \left(\mathbf{g(x,y)}\begin{cases}1, r \geq T_H \\ 0, r < T_L\end{cases}\right)\right]$$

Object Detection and Feature Extraction

After segmenting the image, the pixels of interest are "collected" into objects by a classic depth-first search algorithm. An object is made from segmented pixels, but not all detected objects are fish. Uneaten food, feces, bacteria, algae and even air bubbles can interfere with object detection, because they may appear in the video at any time during a recording. The first stage of analysis which prevents such objects from being detected is size sieving or classification. Like most image analysis software, *AquaObserver* allows the user to select the minimum size (A_{min}) of an object to be identified as an animal. Thus, any object smaller than A_{min} pixels is ignored. The choice of an adequate A_{min} is influenced by all the image manipulations performed before segmentation and by the values of the parameters that affect segmentation. In most of the experiments illustrated here, A_{min} was set between 500 and 1000 pixels.

Another important post-segmentation parameter that affects image analysis, is the between-object gap distance. Recall that segmented pixels are grouped into objects. The between-object gap parameter determines how close a group of pixels have to be from one another to be counted as belonging to the same object. A gap that is equal to 0 (i.e. no gap allowed) means that the animals can come within one pixel of another and still be counted as separate animals. Smaller gaps are preferred, but larger gaps can be used if

the quality of the image requires it. All data reported here was analyzed with a between-object gap of 0.

Measuring fish color

Most published studies use specialized tanks to measure fish color and brightness. Such tanks are fitted with an internal color reference against which the color or brightness of the animal is compared. Because many use crude segmentation algorithms, the researcher must perform extensive tank modifications. For example, the software may require a white background or under-the-tank bottom lighting to cancel shadows. This is because the software uses either image subtraction or color-based thresholds, making it difficult or impossible to distinguish a shadow from a fish.

Still, researchers such as Hoglund (2000) and (Szisch, 2002) have circumvented many of these challenges by using other techniques such as digital still pictures or spectrophotography. However, the techniques used precluded dynamic measurements of color changes within the unpertubed social context in which they often occur. I have performed such measurements with *AquaObserver* and I report my findings in the next chapter.

Solving the problem of uneven illumination

Because of the physical constraints imposed by aquaculture raceways, achieving homogenous illumination is extremely difficult. One of the experimental setup that I used consisted of two sets of lights placed besides the tank. This setup affected the measurements of fish color because as a fish moves around the tank it is not under the same level of illumination in all the frames.

In my next experimental setup, a one-point light source arrangement was used, consisting of 3 lights in a circular configuration and located centrally to the arena, and at the top of the tank cover. Although, with this setup most shadows could be avoided, other unforseen problems arose. For example, the centre was more intensely illuminated than the sides. Another problem that occurred in both setups was that the wall of the tank, being oriented perpendicular to the bottom and perpendicular to the fishes' movement plane, casted a shadow on the animals that swam closer to the wall. Thus, it is difficult to avoid lighting issues in such big tanks. Another possible setup which I did not consider, is to use multiple lights perpendicular to the water surface and equidistant from the centre of the tank and each other. The disadvantage of such setups, besides the difficulty of implementation in an aquaculture facility, is that the animals are now placed under stress from the intense lighting that results. A software solution would proof more useful in the long term.

Therefore, a normalization step to diminish the noise that occurs as a result of the uneven illumination has to be performed for every brightness measurement taken. This brightness correction is performed as follows. A measurement is taken of the color of the tank background at the same position the fish is located on the no-fish background image. This measurement is compared to a user-selected area of the tank in the no-fish background image. The color values of the fish are then adjusted according to the difference between the two background regions. The normalization follows the following formula:

Calculate the average brightness of a randomly chosen area of the screen. This value is calculated ONCE for every video file processed. The user selects this portion of the screen at random, but it most be an area of the arena that has no animals. We called this area the reference.

$$\overline{B}_{reference} = \frac{\sum_{x}^{n}\sum_{y}^{n} f^{red}_{reference}(x,y) + \sum_{x}^{n}\sum_{y}^{n} f^{green}_{reference}(x,y) + \sum_{x}^{n}\sum_{y}^{n} f^{blue}_{reference}(x,y)}{3n}$$

To calculate the next value, the coordinates where the animal is in the arena are used to look at the no-animal background image that is loaded in memory. This background image is used for the background subtraction algorithm. Thus, we can calculate what the brightness of that portion of the screen is and compare it to the randomly chosen reference.

$$\overline{B}_{background} = \frac{\sum_{x}^{n}\sum_{y}^{n} f^{red}_{background}(x,y) + \sum_{x}^{n}\sum_{y}^{n} f^{green}_{background}(x,y) + \sum_{x}^{n}\sum_{y}^{n} f^{blue}_{background}(x,y)}{3n}$$

Now determine the correction factor, *C*, which will be applied to every channel in the segmented portion to be normalized, we find the ratio of the intensity of the reference and the no-fish background:

$$C = \frac{\overline{B}_{background}}{\overline{B}_{reference}}$$

Finally, we use this correction factor to change the brightness of the pixels in the segmented image. Thus, each pixel that makes up the animal is corrected according to the difference between the background and the reference.

Note that this algorithm is applied to each object segmented so that the above calculations are made for each object in the frame. The value of the correction factor will depend on the position of the animal in the frame and the intensity of illumination at that given position in the no-fish background reference frame. A sample of the output of the algorithm is shown in **figure 15**, along with photo inserts that show the actual color of the animal at the point of measurement. Note that in that figure, the color of the two small fish and two bigger fish have been averaged together so that each line represents the color intensity for each of the two size classes (small and big). This was done because

categorizing by size (automatically) is easier and more precise than categorizing by tag color. It was possible to do this, because the two small fish became less dark and the two big fish became darker, at the same time. This is actually not uncommon, and can be better understood by studying the usual behavioral kinematics that lead to color change (**Chapter 2**).

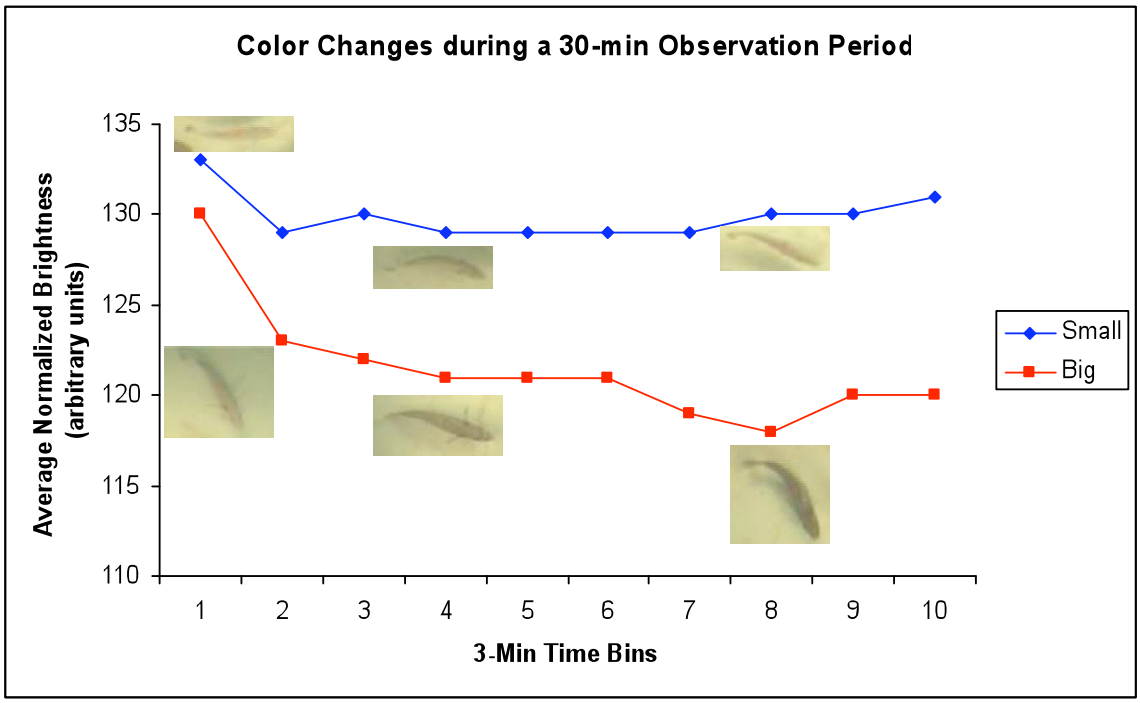


Figure 15. Average intensity of color change as a function of time. Each time bin represents a 3 minute period. The lower the arbitrary unit, the more intensely dark the fish is. The four fish in the tank were split in two categories and the average color change was plotted. Note that when the two big females became darker, the two smaller males became brighter.

Shape Analysis

Detection of *fish entanglements* (fish crossing over other fish, as seen in a 2D perspective) and incomplete objects (fish partially detected because of image segmentation artifacts) can be accomplished in part by *shape-recognition* algorithms. Although a shape-recognition algorithm is still under development, several shape-related

parameters can be currently measured, such as area, perimeter, and average distance from center to perimeter. The area is defined as the total number of pixels that make up an object. The perimeter measurement is based on the *contour*. This contour is obtained by a naïve algorithm based on a contour-following algorithm by Parker (1996). The average distance from center to perimeter is the average of the distance from the centroid of the object to the contour pixels. Using a combination of these shape parameters and some of the texture parameters, the probability of detecting a fish, an entanglement or of a partial detection, can be assessed.

AquaObserver can distinguish between circles, triangles, squares and other simple geometric forms. This simple computer vision ability may be useful in other experiments involving feeding, territorial aggression, or measurements of animal postures.

Pattern analysis: Hu Moments

Hu (1962) defined seven moments that are invariant to translation, rotation, and scaling. These set of moments have been used extensively in computer vision for pattern recognition (Rizon et al., 2006). *AquaObserver* can calculate these seven moment invariants. They may be useful for classification of color changes, as each animal or each sex may have different color changing patterns.

Measuring Texture

Texture is the repeating pattern of pixels that gives a section of an image the appearance of a smooth or rough surface (Review:Tuceryan, 1994). For example, a red balloon has a smooth texture, while a red brick has a rough texture. The texture parameters *AquaObserver* calculates are based on the statistics that describe the distribution of color in a section of an image. Such color distribution histograms can

approximate a normal distribution. Hence, the *normalized central moments* can be calculated and used to describe the texture in an image (Parker et al., 1996; Teh and Chin, 1988). The first three normalized moments are also known as standard deviation, skewness, and kurtosis (Parker, 1996; see also http://en.wikipedia.org/wiki/Central_moment). *AquaObserver* also records the 5th order moment around the mean, which carries information about skew. A color changed fish will show a negative skew, corresponding to an increase in the number of pixels that are *below* the mean brightness level. This negative skew becomes a negative value in the 3rd and the 5th moment.

Automatic and Manual Identification of each animal in the arena

Color tags will be surgically attached to the animals by using a technique that we have developed. The tags used are colored beads brought at a craft store. The beads are roughly spherical in shape with a hole in the middle where the suture is threaded. These tags are attached to the anterior region of the dorsal fin by anchoring it to the bony fin rays. To attach the tag, the fish is first anesthetized in accordance to our IACUC-approved protocols. These beads are not expected to interfere with the normal behavior of the fish.

Detection of colored beads by *AquaObserver* using the full color information and illumination-invariant color ratios is possible. The accuracy of detection is currently very low for certain tag colors (e.g. green and yellow). Red-tagged fish are easily detected, with an accuracy that approaches 100%. The software can be manually programmed to distinguish from up to 16 colored tags or a novel algorithm based on illumination

invariants (i.e. color ratios) can be used to detect up to 8 colors. This novel algorithm is a simple classifier based on color ratios and color intensity.

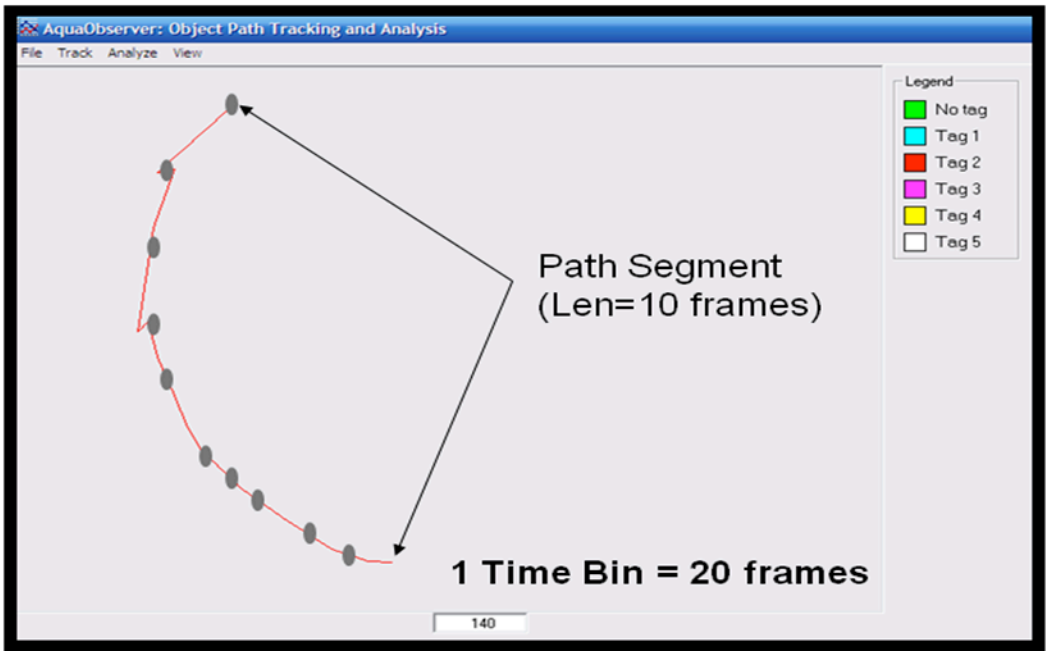


Figure 16. *Path segments are the principal unit of movement measured by AquaObserver's tracking tool. The length of a path segment is equal to the number of continuous frames that an animal was observed before it went off-camera or the algorithm can't detect it. When the detector is set to detect dark color changing fish only, a path segment represents the time that an animal was observed in its dark melanic color state.*

Tracking Animals in the Arena

Tracking is done by an algorithm that considers the displacement of the animal in the X-Y plane, the tag color of the animal, and a calculated probability that the animal in one frame is the same one detected in the previous frame. If, for any reason, the tag id is not available for the last frame, the displacement of the animal is used as the main criteria for tracking. Correct identification of the animal depends on the probability of it being the same animal as in the previous frame. The algorithm assumes that an animal will only move between frames at a speed of no more than its body length/sampling rate.

Hence, some analysis is done after the original image analysis finds the objects and determines their position. This tracking analysis, which I termed *track rebuilding*, uses the positional data contained in a .csv file and creates another .csv file. The researcher can plot the original or rebuilt file to assess how correctly the software plotted the animal tracks.

Positional data from *AquaObserver* is analyzed as *path segments* (**Figure 16**). Path segments represent a continuous, recorded trajectory of an object in the arena. The rebuilding algorithm generates these path segments. The rebuilding of track file data is a very important step in the positional data analysis because during rebuilding, an artificial intelligence algorithm assigns each object detected to a path segment. The algorithm relies on several object parameters, such as the object's position and trajectory, the tag color of the animal as detected by the tag identifier algorithm, and various other physical parameters such as the object area and shape descriptors, to establish the identity of the object. Once the object identity is confirmed, a confidence probability is calculated for that point in the segment, and the object is assigned to the segment.

When the object detection fails to detect an object in one frame but succeeds in the following frame, the algorithm is able to "join" the object to the path segment it belongs. For every object detected, *AquaObserver* generates an entry in a table. In this table, the rebuilding algorithm keeps count of how many frames an object that was detected is no longer detected. An objects unique identification number (UIN), and its entry in the table, will persist only for a determined number of frames, unless the object is positively identified again in another frame. If the object is no longer identified, it is deleted from the table. This can occur if a fish swims out of the frame or if a fish swims

too close to another animal. In the later case, the area of the object increases (two animals vs. one) and a new UIN is assigned. The information in the rebuilt file should clearly show that the objects have "joined".

AquaObserver can display track file data using colored lines to identify each object. To ensure correct detection, the user can select to display the tracks overlapping with the video file.

Determination of residence time

The arena is divided into two imaginary zones: a left zone and a right zone. The track file is analyzed to assess the number of times each animal was observed in each zone. Each track file can be sub-sampled at determined time periods so that the data shown can be correlated to other behavioral events. For example, we have determined that in seabream, color changing behavior is related to territorial behavior (**Figure 17**).

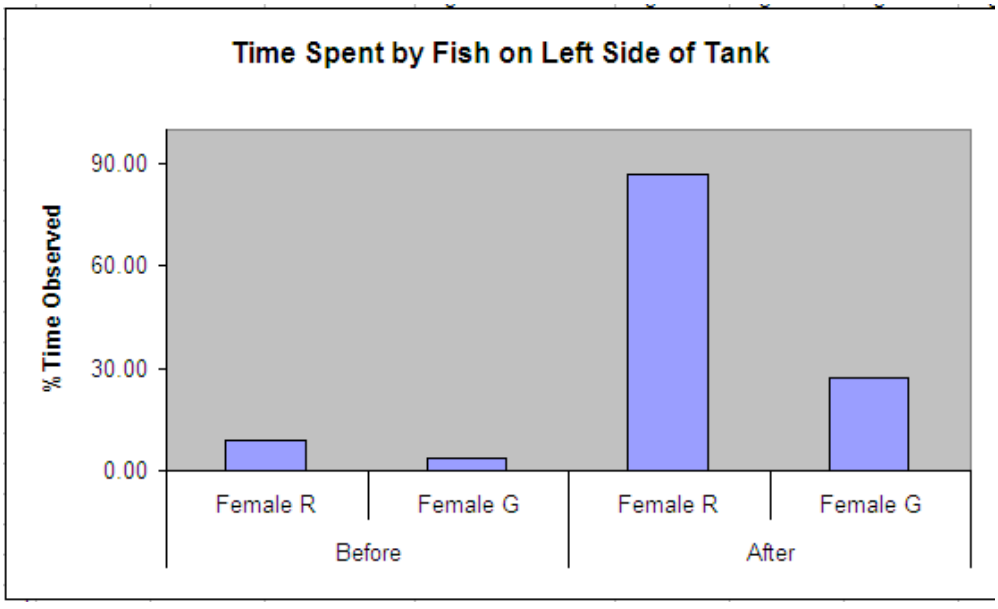


Figure 17. Residence time on the left side of the tank, for a Red-tagged female and a Green-tagged female seabream, before and after a strong color change (in both fish). The Red-tagged female spent about 90% of the time in the left side of the tank, once the color change occurred. The Green-tagged female spent 70% of the time in the right side of the tank.

Conclusion

To provide a more comprehensive analysis of Gilthead Seabream behavior, I have developed a set of tools using VB6. These software tools enable the ranking of fish in putative dominance hierarchies, determination of the hierarchy's linearity, and also allow the experimenter to construct ethograms, analyze behaviors as Markov Chains, and study group structure as a function of rank stability. Bo th group and individual behaviors can be studied. Furthermore, the software allows for the measurement of fish melanic color while the fish is actively swimming, without the need of specialized hardware or tank modifications that could impede normal behavior. By using advanced computer image analysis techniques, the software is able to correctly quantify the magnitude of these color changes, providing an innovative technique for the measurement of physiological color changes in fish which, to my knowledge, has never been used before. Using all of these tools, I will explore the behavior of the Gilthead Seabream and how it may influence sex change in this species.

Apendix A: Derivation of the BAS Filter

Color ratios provide information about the color represented on the RGB space. The information encoded by color ratios is usually invariant of illuminant intensity. Thus, color ratios are useful for segmenting color images. However, implementing an algorithm that works on each of the three RGB channels is not easy and I have found that adjusting the thresholds using ratios is difficult and time consuming. Here, I have proposed a simple approach to this problem that works well for images with uneven illumination and requires setting only two thresholds.

We begin by defining two sets of full color (RGB) images: a **reference** (arena with no animals), and the **frame** image that we would like to segment. Each of these two images will be defined by the following functions:

$Red_{frame}(x,y)$, $Green_{frame}(x,y)$, $Blue_{frame}(x,y)$ = *Frame image to be analyzed*

$Red_{reference}(x,y)$, $Green_{reference}(x,y)$, $Blue_{reference}(x,y)$ = *Frame with no animals (empty arena)*

If we want to obtain the color ratios that define these images, we need to calculate only two ratios, a red/blue and a green/blue. First, we can calculate the ratio of red to blue for *each* pixel in the frame:

$$R_{frame(R:B)} = \frac{\mathrm{Red}_{\mathrm{frame}}(x,y)}{\mathrm{Blue}_{\mathrm{frame}}(x,y)}$$

Then we calculate the ratio of green to blue for *each* pixel in the frame:

$$R_{frame(R:B)} = \frac{\mathrm{Green}_{\mathrm{frame}}(x,y)}{\mathrm{Blue}_{\mathrm{frame}}(x,y)}$$

To implement the BAS filter, we need to compare the frame image to the reference image. If the color ratios are the same, then the segmented pixels most likely belong to the background. Therefore, we need to calculate these same ratios for the reference image too. Again, we calculate the ratio of red to blue for each pixel in the reference:

$$R_{reference(R:B)} = \frac{\text{Red}_{\text{reference}}(x, y)}{\text{Blue}_{\text{reference}}(x, y)}$$

Then, we calculate the ratio of green to blue for each pixel in the reference:

$$R_{reference(G:B)} = \frac{\text{Green}_{\text{reference}}(x, y)}{\text{Blue}_{\text{reference}}(x, y)}$$

If the ratio of green to blue in the frame and the ratio of green to blue in the reference is the same, then the ratios of these two ratios would equal one. We therefore define two more ratios:

$$R_1 = \frac{R_{frame(R:B)}}{R_{reference(R:B)}}, \text{ and } \quad R_2 = \frac{R_{frame(G:B)}}{R_{reference(G:B)}}$$

Once more, if the ratio red to blue and the ratio of green to blue in both the frame and the image reference are equal, then both R_1 and R_2 should be approximately equal to 1. Ignoring noise, the product of both ratios should be approximately 1 if the images have the same color in the same pixel. We make the product of these two ratios the value of the BaS filter and we apply a low and a high threshold for it. Therefore, we define R_{BAS} as:

$$R_{BAS} = R_1 \times R_2$$

Simplifying this equation yields the following formula:

$$R_{BAS} = \frac{\left(Blue_{frame}(x,y)\right)^2 \times Green_{reference}(x,y) \times \mathrm{Re}\, d_{reference}(x,y)}{\left(Blue_{reference}(x,y)\right)^2 \times Green_{frame}(x,y) \times \mathrm{Re}\, d_{frame}(x,y)}$$

After using this equation for our images, we realized that increasing the weight of the blue channel yielded consistently more accurate segmentation of the animals without inclusion of the fish. The following is the actual equation we used for segmenting images in *AquaObserver*:

$$R_{BAS} = \frac{\left(Blue_{frame}(x,y)\right)^3 \times Green_{reference}(x,y) \times \mathrm{Re}\, d_{reference}(x,y)}{\left(Blue_{reference}(x,y)\right)^3 \times Green_{frame}(x,y) \times \mathrm{Re}\, d_{frame}(x,y)}$$

Pixels are segmented according to the following criteria:

$$g(x,y) \begin{cases} 1, R_{BAS} \geq T_H \\ 0, R_{BAS} < T_L \end{cases}$$

Therefore, the BAS filter requires only two parameters. If all the ratios calculated are equal, then $R_{BAS} \approx 1.0$. The low-threshold (T_L) and high-threshold (T_H), determine how different the background reference segment must be from the frame image segment in order to be accepted as a pixel belonging to a region of interest.

Chapter 2: Seabream behavior and dominance hierarchies during two different stages of gonad development.

Introduction

In many gregarious and shoaling species, aggressive interactions and competition for limited resources often lead to the establishment of dominance hierarchies (Wilson, 1975). An individual's rank in the hierarchy determines its ability to access food, territory, and other resources. In wrasses and clownfishes, the sex change order is strongly correlated to the rank of an individual in the group (Fricke and Fricke 1977; Warner and Swearer 1991). Indeed, a corollary of Sex Allocation Theory is that dominance behavior can be a function of sex (Ghilselin, 1969; Allsop & West, 2004; Munday et al., 2006). Accordingly, if males invest more energy in reproduction than females, they should dominate the social group and *vice versa* (Charnov, 1982). Conversely, if SAT applies to sex change, then those individuals that have more energy to invest in reproduction might become females or males, depending on the specific life history of the species.

Pelagic and benthopelagic hermaphrodites such as groupers, sparrids, and related species, are of great economic importance (Pomeroy et al. 2004; Piumsombun et al. 2005). Unlike reef fish, the relationship between behavior and sex change has not been explored in pelagic species. In contrast to reef dwellers, most are broadcast spawners that have a distinct spawning season and usually migrate to and from foraging and spawning grounds (D'Ancona 1948; Chaoui et al. 2006; Pears et al. 2007; Wu et al. 2008). Adults can achieve larger size than reef fishes and therefore many, such as seabream, are economically important food fish that are farmed at large aquaculture operations. Often,

after several years the broodstock develops an over-abundance of the terminal sex and fish have to be hormonally or socially manipulated to obtain an appropriate sex ratio (Zohar et al., 1995; Shinn-Lih et al. 2003). Knowing what social factors determine the outcome of sex change is imperative for fisheries managers (e.g. Davey 2005) because in the wild, selective fishing of the older or bigger animals can shift the sex ratio of the population (Aguilar-Perera 2007; Hamilton et al. 2007). Thus, the question of how sex ratios are established in these species should also be addressed (Benton 2005).

Gilthead Seabream is a protandrous seasonal spawner from the Mediterranean Sea (D'Ancona 1948). It is an excellent model for studying hermaphroditism, because its gonadal cycle has been well characterized at the anatomical level (Zohar et al. 1978; Zohar et al. 1984) and the molecular level (Wong and Zohar 2004; Wong et al. 2006). Size or age determines if a male changes sex, but the exact way in which sex is decided is still unknown. In the Mellah lagoon on the coast of Algeria, the population of Gilthead seabream shows a sex-skewed size distribution, which is consistent with a protandrous hermaphrodite that changes sex after reaching 44.0 cm or more (Chaoui et al. 2006). Indeed, in captive seabream, males are precluded from changing sex by the presence of larger and older females (Happe and Zohar, 1986). However in both of these studies, age and size are confounding factors. Furthermore, behavioral interactions between females and males have never been studied. Thus, what cues triggers sex change and whether these cues are visual, behavioral, pheromonal or consist of some other factors or combination of factors is still unknown.

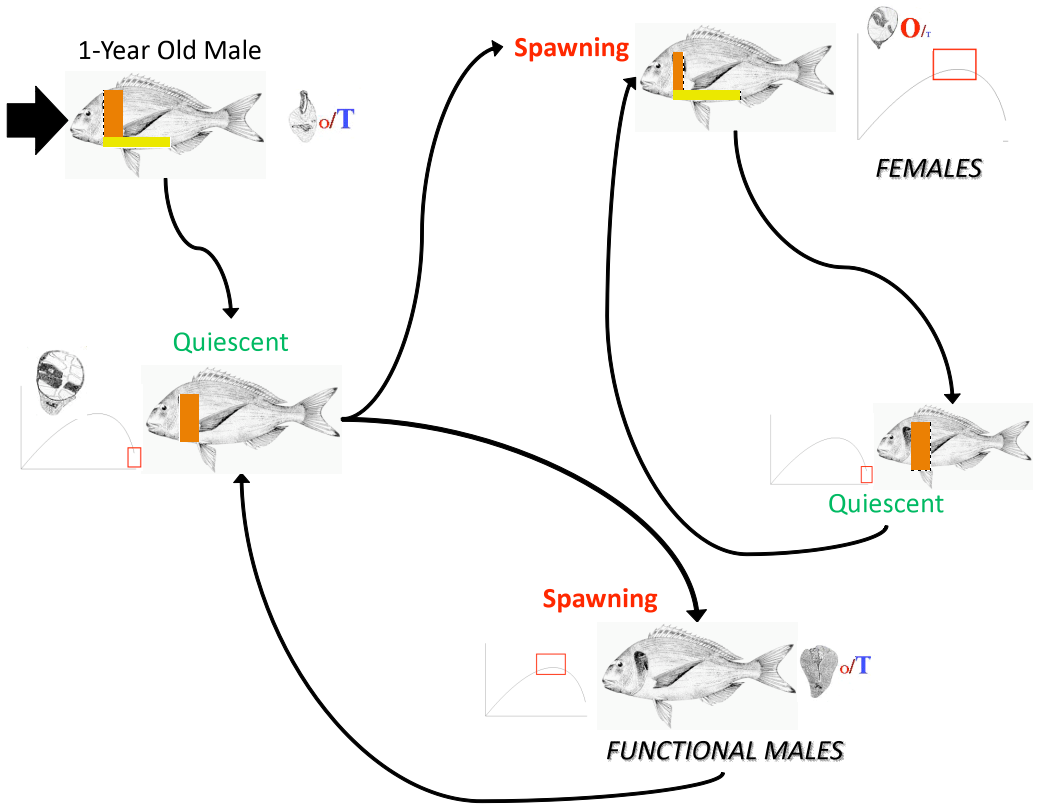


Figure 18. *The complete gonad cycle of the Gilthead seabream. Gonad development and Estradiol levels are shown in the inserts. The intensity of the pre-operculum orange color and the abdominal yellow band are also represented. Gonad diagrams and gonad pictures taken from Zohar et al. (1984).*

To understand the influence of behavior on sex change, it is important to understand other factors that in turn influence behavior. For example, behavior itself could be influenced by the sex of the animal or the stage of gonad development. Since seabream is a seasonal spawner, its gonad undergoes multiple stages of development and regression (Zohar et al. 1978). For simplicity, we consider gonad development in this fish to consist of four distinct seasons (see **Figure 18**): pre-spawning (August-November), spawning (December-March), and a quiescent stage, with an earlier, post-spawning quiescence consisting of quick gonad involution (April-May) followed by complete quiescence (June-July). During quiescence, gametogenesis is arrested, the

gonad is not producing significant amounts of steroids and the fish is not in a reproductively active stage (Wong et al. 2006). Before entering the pre-spawning gonad development, there is a period in which the ovarian portion of the gonad undergoes some development but no testicular development is observed. This development, as occurs in other stages of this cycle, seems to be asynchronous, with each individual in a group of animals at a different stage of involution or development. It is during this asynchronous development of the ovarian portion of the gonad when we believe that the sex of the animal is decided. Later, during the pre-spawning and spawning stages, the gonad of the fish begins to differentiate and becomes mature, gametogenesis restarts and the gonads begins to produce significant amounts of steroids (Zohar et al. 1978; Wong et al. 2006). The gonad steroid profile is different for males and females during these two stages (Wong et al. 2006; Also see **Chapter 3**).

Because of these different gonad steroid profiles, the intensity of aggressive behavior is expected to vary with sex and gonad development. Why? In most teleost species, the presence of androgens increases territorial behavior and aggression. The P_{450} aromatase enzyme complex mediates the conversion of androgens to estrogens. Aromatase is present in both brain and gonads. Aromatase activity in the fish brain is inversely related to aggressive behavior in at least one species of sex-changing fish (Black et al. 2005). Thus, during spawning aggressive behavior could be increased or decreased, depending on the species.

The following series of experiments will explore the basic behavioral mechanisms by which sex change could be influenced in the Gilthead seabream. Since there was no information about the captive behavior of seabream, I provide here the first detailed

account of agonistic interactions among seabream fish. The accounts are both descriptive and quantitative. I also address the dynamics of group and hierarchy formation. Then, I directly address the question of whether seabream males or females are more aggressive. Finally, I test the hypothesis that the gonadal cycle can affect the expression of aggressive behaviors. The effects that these changes have in the dominance hierarchy are also explored. Outlining the basic behavioral profile of seabream throughout its gonad cycle and determining male and female specific behavior, are among the objectives of this work. In fact, the findings discussed here lay the foundation not just for this work, but for any other work involving the behavioral influences of sex change in seabream.

METHODS

Experimental Animals

Seabream, *Sparus aurata* is native to the Mediterranean Sea. The animals used in this experiment where hatched at indoor facilities in Baltimore, Maryland. The fish were kept in tanks of $13m^3$ before the experiment began. When the spawning season began, fish from these tanks where sexed, and the necessary number of females and males where obtained from this tank and transferred to a smaller $2m^3$ tank which shared the same water and filtration system as the filming tank. Care was taken to match the temperature and salinity in this dual $2m^3$ system to the temperature and salinity in the stock $13m^3$ system.

Dyad Encounters

A total of 13 animals were used in a series of dyad encounters in which two animals were used twice in separate dyads but with different adversaries (**Table 2**). The experiments were carried out in a $2m^3$ diameter tank divided into four equal compartments by two tank dividers. Before the encounter began, four fish from an

identical undivided $2m^3$ tank were transferred into the experimental tank and tagged with one or two Floy tags. Each fish was placed in a separate compartment, and the fish were isolated for a period of 5 days to negate the effect of previous encounters or social experience. The dyadic encounter began when one of the barriers was removed to allow the pairs of fish to interact. The other barrier remained in place to isolate each dyad visually from each other. Thus, two dyads could be run in same tank. This was done

Dyad ID	Date	Sex	TTL Length (cm)	PIT
1	11-Mar-03	**M**	31.5	0E59
1	11-Mar-03	**F**	32	2F43
2	11-Mar-03	**M**	26	474F
2	11-Mar-03	**M**	31.5	7948
3	24-Mar-03	**M**	31	6303
3	24-Mar-03	**F**	30	2741
4	24-Mar-03	**F**	32.5	1543
4	24-Mar-03	**F**	27.5	727E
5	3-Apr-03	**M**	32	0E59
5	3-Apr-03	**M**	30.5	1B78
6	9-Apr-03	**F**	29.5	1920
6	9-Apr-03	**M**	32	7948
7	5-May-03	**M**	30	7027
7	5-May-03	**F**	29	7D6A
8	5-May-03	**F**	30	7B4A
8	5-May-03	**F**	33.5	052A

Table 2. Sex and size of each fish used in the dyadic encounters. Note that each individual of the pair of fish observed has the same dyad ID. Thus, a total of 8 pairs were observed.

because tank resources are limited, and because it intensified the aggression by making the arena smaller, thus forcing fish to swim in a more confined space and increasing the pair's encounter rate. Each pair was observed several times during the course of 3-5 days for a period of 30 minutes. For each observation, the fish that had the most aggressive

attacks was designated the winner. A tie was declared when both fish initiated the same number of aggressive behaviors. These observations were done by the observer standing a few feet from the tank, being careful not disturb the fish.

SPAWNING **QUIESCENT**

Feb-23-05

PIT	SEX	L	W	GW
0340	Male	28	0.37	3
1947	Male	25	0.25	2
0B21	Female	43	1.28	33.7
6970	Female	42	1.29	57.5

May-3-05

PIT	SEX	L	W	GW
5322	Male	28	0.4	
3B1E	Male	30	0.46	
795C	Female	38	1.01	0.02
1920	Female	38	1.07	0.02

March-11-05

PIT	SEX	L	W	GW
2F5C	Female	37	1.25	29.4
0B21	Female	42	1.46	43.1
6970	Male	26	0.3	0.08
0F47	Male	27	0.35	0.45

June-30-05

PIT	SEX	L	W	GW
1920	Male	29	0.4	0.88
7D6A	Male	30	0.5	2
510A	Female	42	1.45	19
454A	Female	40	1.25	18.9

Table 3. Sex, Length (L) in centimeters, Weight (W) in kilograms, and Gonad Weight at sacrifice (GW) in grams. At the end of the experiment, the animals were euthanized in order to perform additional molecular biology assays on the brain tissue. For each group, the date of the beginning of the observation (formation of tetrad) is shown.

Two types of dyads were carried out: A size-controlled dyad where sex was the experimental variable (sex bias) and a sex-controlled dyad where size was the experimental variable (size-bias). In the dyad where the size was not the experimental variable, both male pairs (n=2) and female pairs (n=2) were used and size was controlled by attempting to match pairs with a difference of less than 5% fork length between each

fish. In the size-bias dyads, the fork length difference between paired fish was greater than 10%. A total of 8 pairs were observed, four were size-matched dyads and four were sex-matched dyads.

Tetrad Encounters

We observed four tetrads of *S. aurata*. Each tetrad was observed during a specific period of their gonad cycle. Two "spawning" (S) groups were observed during February-March. Two non-spawning (NS) or quiescence group were observed during May/June (**Table 3**).

Before transferring the animals to the observation tank, each fish was placed in a small tank with 0.02% phenoxyethanol. After the fish was in deep anesthesia, the sex of the animal was determined by gently pressing on the belly of the fish to release its gametes (eggs or sperm). A radio-frequency passive transponder tag (PIT, Northwest Marine Fisheries) was placed intramuscularly between the dorsal fin and the head of the animal. To identify the fish visually in the video-recordings, two small beads where attached to the dorsal fin, using a surgical thread. This tagging method was developed by our lab in conjunction with COMB's aquatic veterinarian. To identify all four animals in the videos, two size classes and two different color tags where used. The color beads used were green or red. In the spawning groups, two big female *S. aurata* where placed together with two small males. In the non-spawning groups, two big regressed "females" were placed with two regressed "males". At the termination of the experiment, the animals were euthanize and the brain was frozen in dry ice in order to perform additional molecular assays (**see Chapter 4**).

Video Recordings

Recordings were performed using a standard analog video camera attached to a computer system. A $2m^3$ circular vessel with an open top was used as the recording arena. All videos were directly digitized by a video capture card (Winnov, USA) to a Windows PC. A completely automated video recording system based on the software included in the Winnov Sofware Development Kit was developed to record each observation at the specified time interval and for the specified time. Each 30 minute recording period took place at the following programmed times: 0930 H, 1100 H, 1230 H, 1400 H, and 1530 H.

Using *AquaObserver* video player and behavior logger, we observed a total of 126 hours of video, 64.5 hours of video from two non-spawning groups and 61.5 hours of video from two spawning groups. We analyzed a total of 252 log files.

Definition of Behaviors

The following definitions will be used for this behaviors based on Carruth (2000). A chase occurs when a fish changes its swimming direction and speed in response to a sudden approach by another fish. A nip is defined in a similar way, but the attacker may already be in close proximity to the attacked fish and the attacker adopts an "open mouth" posture (OM). The frontal aggression also has an "open-mouth" posture, but both fish are in the same posture. As the name implies, the fish adopt a head-to-head (HH) posture by facing each other. The attacker in a frontal aggression is defined as the fish that moves forward, while the fish that "reverses" is the attacked fish. In many instances where this behavior occurs, it is followed by a change in body coloration intensity (CC) in one or both of the animals involved

Table 4. Behaviors used in the analysis of dominance and behavior sequences. These are the most commonly observed behaviors in captive Gilthead seabream.

Behavior	Code	Description	Relative Frequency
Chase	*Chase*	Attacker rapidly swims towards recipient. Recipient responds by quickly swimming "out of the way", but attacker persists for a few seconds.	Very common; Year-round.
Nip	*OM*	Attacker approaches recipient, opens mouth (not always visible); Recipient swims away, but attacker does not pursue.	Very common; Year-round.
Frontal	*HH*	Initiator swims towards recipient, head-on and facing it. Attacker "pushes" forward, while the lose swims backwards. One fish disengages attack by swimming away or attacking.	Not frequent. Year-round. Hard to score winner.
Color Change	*CC*	A fish changes to a darker or lighter color intensity, usually in a very specific pattern.	Common but occurs more during intense fighting. Occurs year-round.
Spawning Chase	*SPW*	A female spontaneously begins to swim fast without a fish behind it. Another fish (male) "joins" and chases the first female. The female swims closer to the surface. The male often swims at an angle and impacts the surface, then swims back to the bottom of the tank. The process is repeated many times.	Infrequent. Occurs only during spawning period, when it becomes frequent. Mostly observed in February and not March.

In the spawning groups, when typical spawning chases were observed, they were also recorded in the behavior log as spawning chases (SPW) so as not to be confused with regular aggressive chases. Spawning chases were characterized by rapid swimming of the female close to the water surface, closely followed by a chasing male who usually swam beneath the spawning female. In some instances the male was observed quickly "hitting" the water surface after the female had left, an action that may involve the release

of the gametes by the male. Most spawning chases were observed in February, during the peak spawning season (data not shown). No further analysis was attempted, and the remaining of this text will refer to a chase only in the context of aggression unless otherwise specified. Only chases and nips are used in the dominance analysis. These behaviors are summarized in **Table 4.**

Statistics

Behavior frequency data was analyzed using a chi-square test with Yates correction only applied when needed. Rank data was analyzed using non-parametric methods, such as the Mann-Whitney U test, and the Wilcoxon-signed rank test. ANOVA's and t-test were applied as needed. The p-value was set to 0.05 in all test. Special tests that were designed for specific sociometric parameters, such as stability index measurements, were also applied but their implementation is described in Chapter 2. For the Markov-chain analysis, the inactivity period was defined as two times as long as a time bin. The time bin resolution was set to 30 seconds. Thus, the analysis looked back to the log activity one minute before and after each behavioral event.

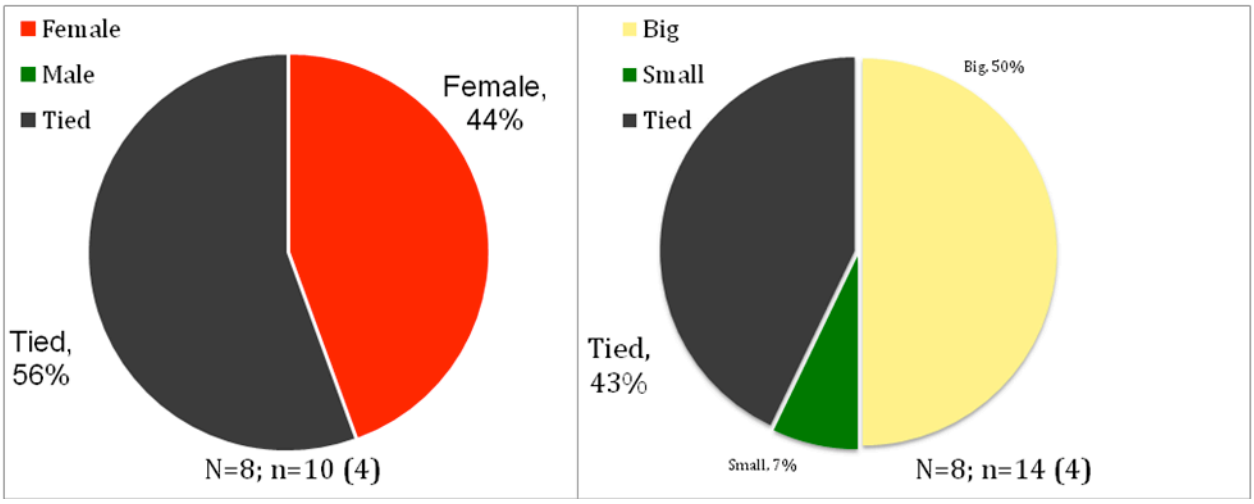


Figure 19. Results of male/female (A) and male/male and female/female (B) dyads. The results are shown as percent of individuals that "won" the dyadic encounter during an observation from total observations. N is the number of animals, n is the number of observations, and the number in parenthesis is the number of dyads analyzed.

RESULTS

Dyad Encounters

Males did not win a single encounter when they were size-matched in the four dyads observed (**Figure 19**). Females won most of the individual observations (Females=44%), but in most the results was a tie (Tie=56%). The bigger fish won over the smaller fish in 7 out of 14 observations (Big=50%) of the four dyads, but in one occasion the small fish was observed to behave more aggressively than the bigger fish (Small=7%). However, in many of these observations, both fish were equally dominant (Tied=43%).

Sex and size bias in Tetrad encounters

When all individuals are considered together, regardless of gonadal development, mature males and regressed males initiated most of the aggressive behaviors observed (data not shown; t_{69}=-3.46, p<.001). However, there was no sexual bias in the number of attacks received by males or females (t_{120}=1.25, p=0.11). Malse also held most of the higher

(more dominant) ranks (**Figure 20**; Mann-Whitney: U_{12}=51, p=.045). However, because of the way the experiment was designed, these males where also the smallest and youngest in the groups. I consider this aspect of the experiment later, when I discuss the results.

The May tetrad was unusual in that all the animals showed higher frequency of aggressive behaviors compared to the other three groups (ANOVA Single Factor: F_3=29.57, p<.001). Regardless of spawning status, there was no correlation between the size of the animal and the number of aggressive acts (Data not shown; Total body length vs. Aggressions, R^2=0.03).

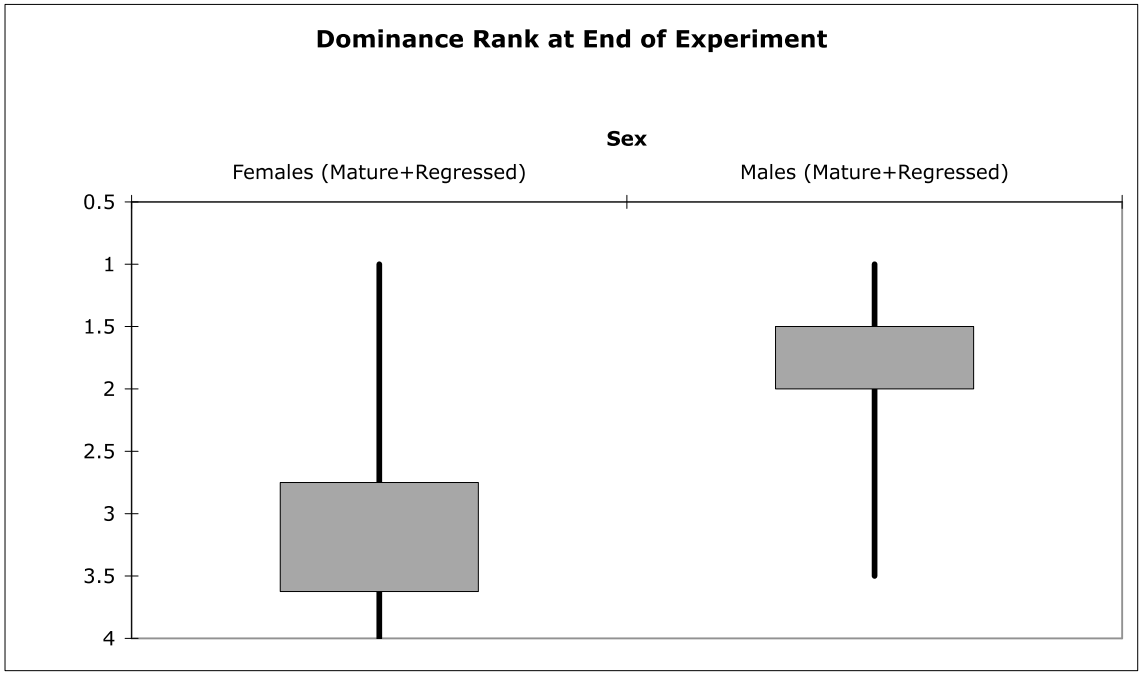


Figure 20. Box and Whisker plot showing the ranks of males and females from all the experimental groups. The median rank of females was 3.5, while the median rank of males was 2. There is a significant difference among these two groups (Mann-Whitney: U_{12}=51, p=0.045)

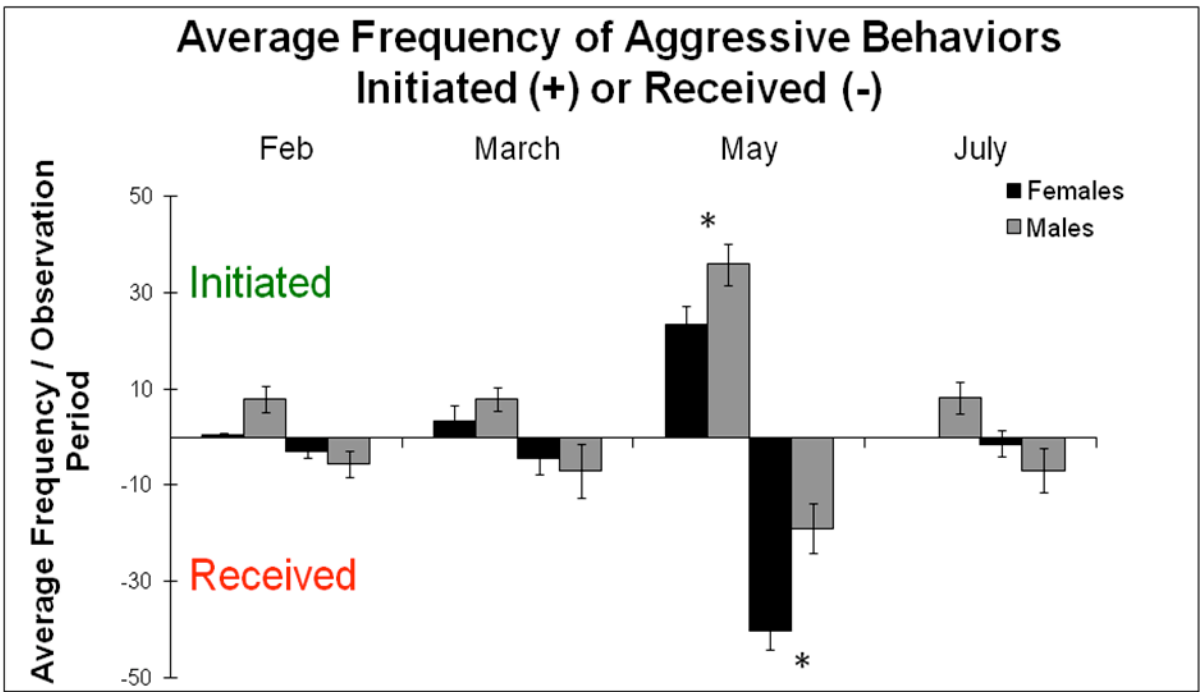


Figure 21. Frequency of initiated or received behaviors. In May, a non-spawning group, the difference in initiated or received aggressions was significant between males and females. Males initiated most of the aggressions during this month (t_{40}=-2.06, p=0.045), while females received most of the aggression (t_{40}=3.62, p <<0.01).

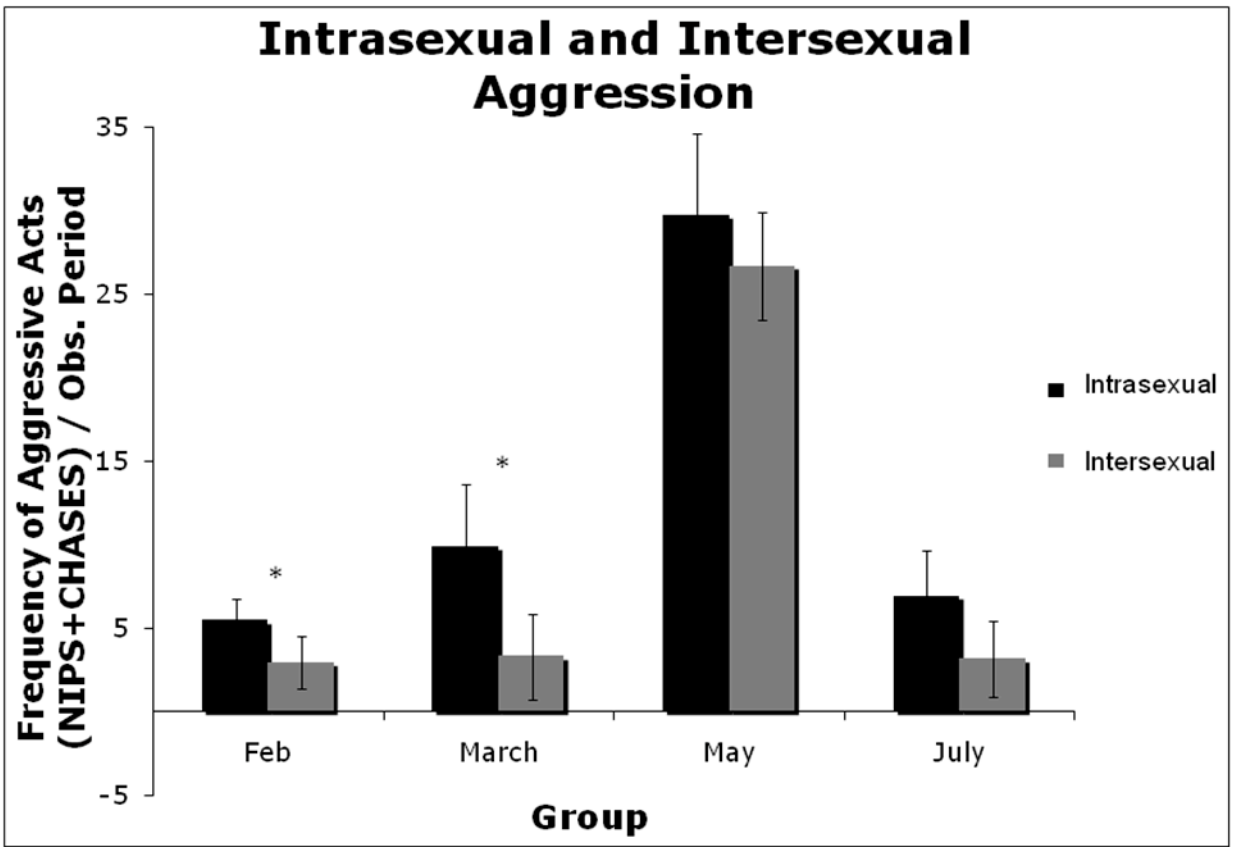


Figure 22. Intrasexual and Intersexual Aggression among groups of spawning and non-spawning Tetrads. February and March are spawning groups while May and July are quiescent groups. Intrasexual aggression was significantly higher only during the spawning months (Fig 3. Feb: t_{17}=2.74, p <<0.01. March: t_{17}=2.20, p =0.02. May: t_{20}=0.47, p =0.32. July: t_{20}=1.88, p =0.04).

Spawning Groups

The two spawning groups observed showed similar levels of average aggressive activity per observation period (**Figure 21**). All individuals in each of the two spawning groups showed aggressive behavior. In February, during the peak of the spawning season, males were more aggressive than females (**Figure 21;** t_{17}=-2.63, p=0.018). Aggressive behavior among fish of the same sex was always higher than among fish of different sexes (**Figure 22**). We observed these animals for a period of 10 days and analysis of this data showed that a weak hierarchical structure formed. However, these spawning tetrads showed no more than 3 unique ranks, suggesting a lack of linearity in the structure

(data not shown). This was confirmed by calculating the Landaus Index H for each day of observation (**Figure**), which showed that for both groups the median Landau's Index was 0.2, suggesting that a linear hierarchy did not form.

Quiescent Groups

During the month of May, just before sex change is thought to begin, all individuals showed increased aggression (**Figure 22**). Regressed females initiated almost as many attacks as the regressed males, but males initiated more aggressions than females (**Figure 21;** t_{40}=-2.06, p=0.045). Females were the recipients of most of these attacks (**Figure 21;** t_{40}=3.62, p <<0.01). Furthermore, the May group was the only group we observed in which aggressive interactions among fish of different sex, occurred with identical frequency as aggressive interactions among fish of the same sex (**Figure 22;** Feb: t_{17}=2.74, p <<0.01. March: t_{17}=2.20, p =0.02. May: t_{20}=0.47, p =0.32. July: t_{20}=1.88, p =0.04). As can be observed from the results of this statistical test, July showed a similar tendency but the difference between intrasexual and intersexual aggression in this group was still significant at the 0.05 level. The elevated intersexual aggression was mostly due to an increase in the aggressive behavior of males towards females (data not shown).

In the two quiescent groups, the two regressed males where constantly bidding for the top spot in the hierarchy (**Figure 23**). In one group, the regressed males switched rank every observation and data for both groups showed that the number of ranks in the group was usually 2-4.

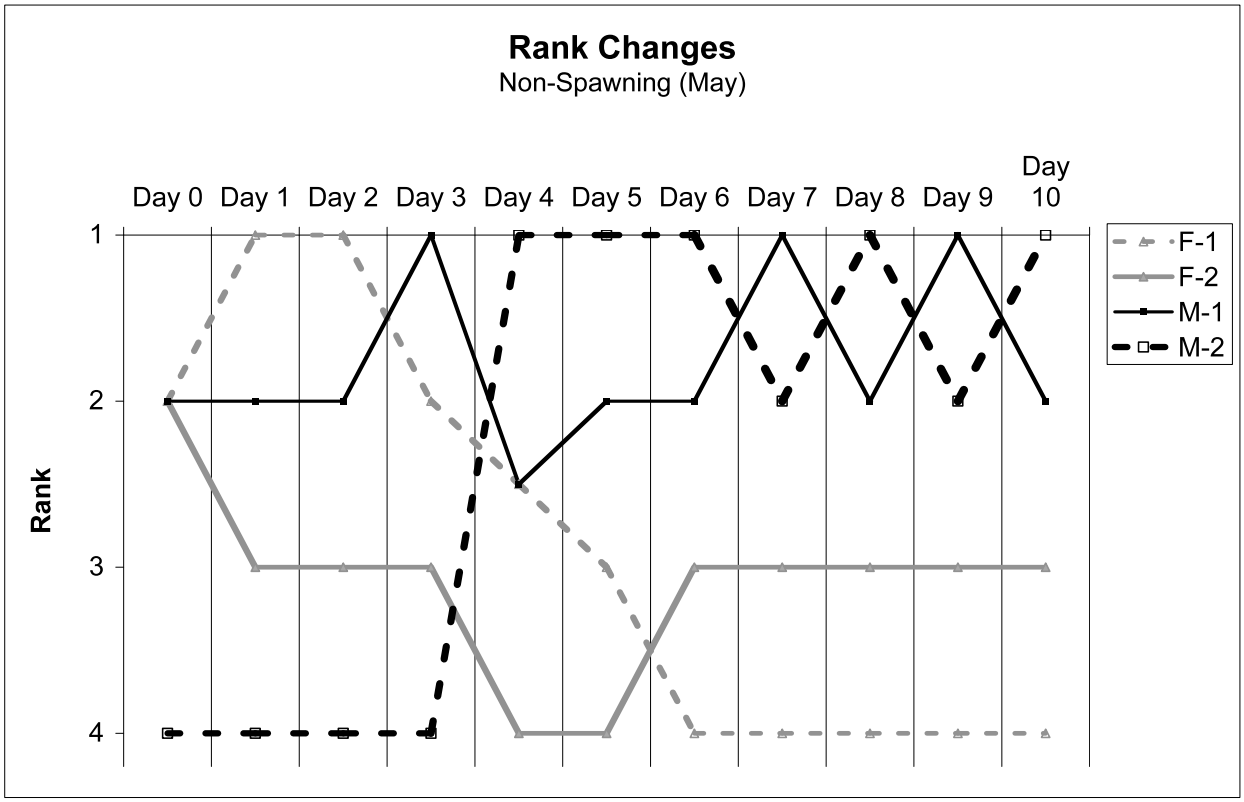


Figure 23. Daily dominance ranks among animals in a Non-Spawning group (May). Ranks are calculated by AquaObserver's ranking algorithm. F-1 and F-2 are fish that last spawned as females (terminal sex), while M-1 and M-2 are regressed males.

Linearity and Instability of Hierarchy

Fish in the quiescent groups showed heightened aggression. Our analysis of this contrast to what we observed in the spawning groups, in which most of the observation periods were characterized by a hierarchical structure with "shared" ranks. Analysis of the behavioral observations should yield four ranks, one for each animal. Indeed, this is what was observed in the quiescent groups: each animal was assigned a unique rank by the algorithm.

Where "shared" ranks existed, non-linear hierarchies are suspected because those hierarchies would tend to be less steep. This suggested the existence of a linear hierarchy in the quiescent groups and a non-linear hierarchy in the spawning groups. I

confirmed this by calculating the daily Landaus Index based on calculations of rank for each day (**Figure 24**). All groups began with a highly disorganized and non-linear group, which became more linear after day 5 but only in the quiescent groups. When all observations are grouped, in the spawning groups, linearity was low (Feb. median H=0.55 and average H=0.52; Mar. median H=0.60 and average H=0.46), while in the quiescent groups, linearity was higher (May median H=0.75 and average H=0.75; Jul. median H=0.65 and average H=0.68).

Both spawning and quiescent groups showed higher instability between days 1-5 (**Figure 25**). This occurred because the animals changed rank a number of times during these days (see **Figure 23**). Interestingly, average instability was less for the quiescent groups than the spawning groups (S_{Feb}=0.19 and p=0.30; S_{Mar}=0.15 and p=0.12; S_{May}=0.12 and $p<<0.01$; S_{Jul}=0.23 and p=0.04). To interpret these results, consider that the p value is calculated by computing all or most of the possible order of observations that would yield an S value lower than the observed value (refer to Chapter 2). Thus, it establishes how significant the observed S is or how stable the hierarchy is on average, regardless of the order of observations. The lower the p, the more stable the group is.

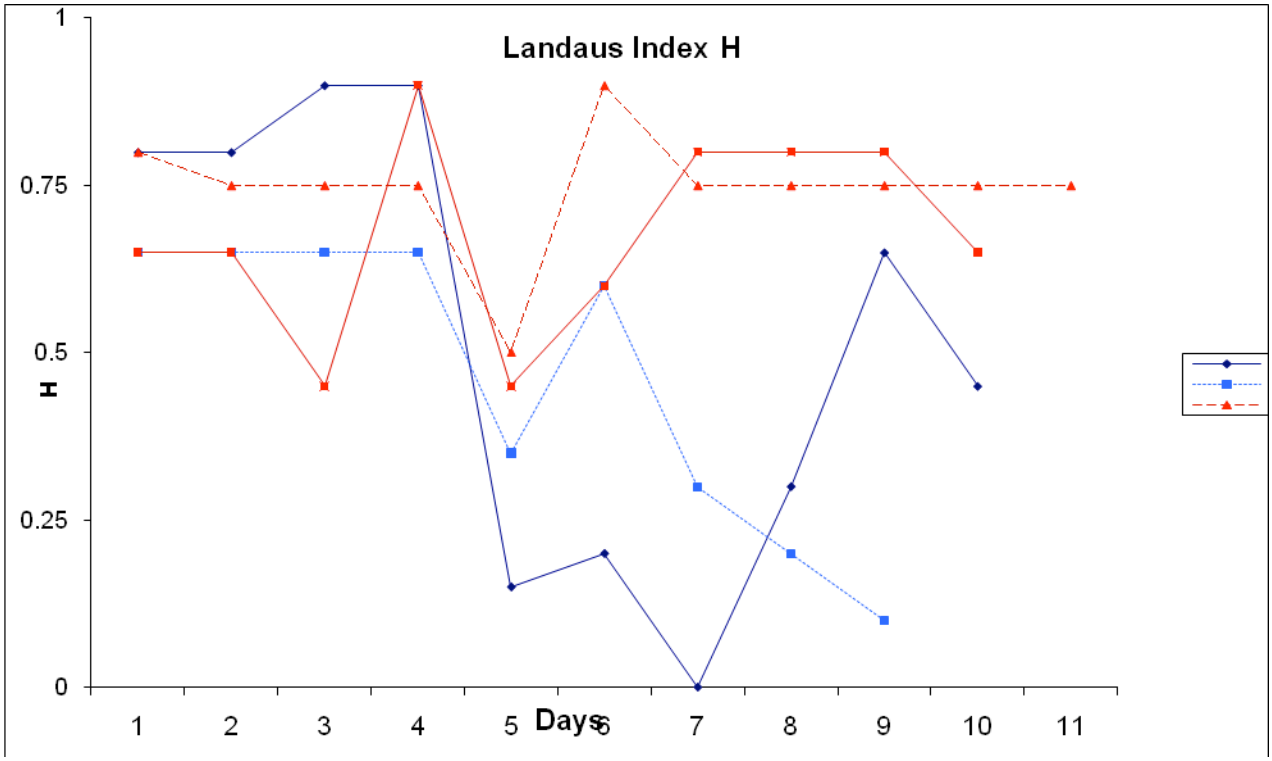


Figure 24. Daily changes in Landau's linearity index (H) during the observation period. At the end of the dyad, linearity was higher for the summer groups.

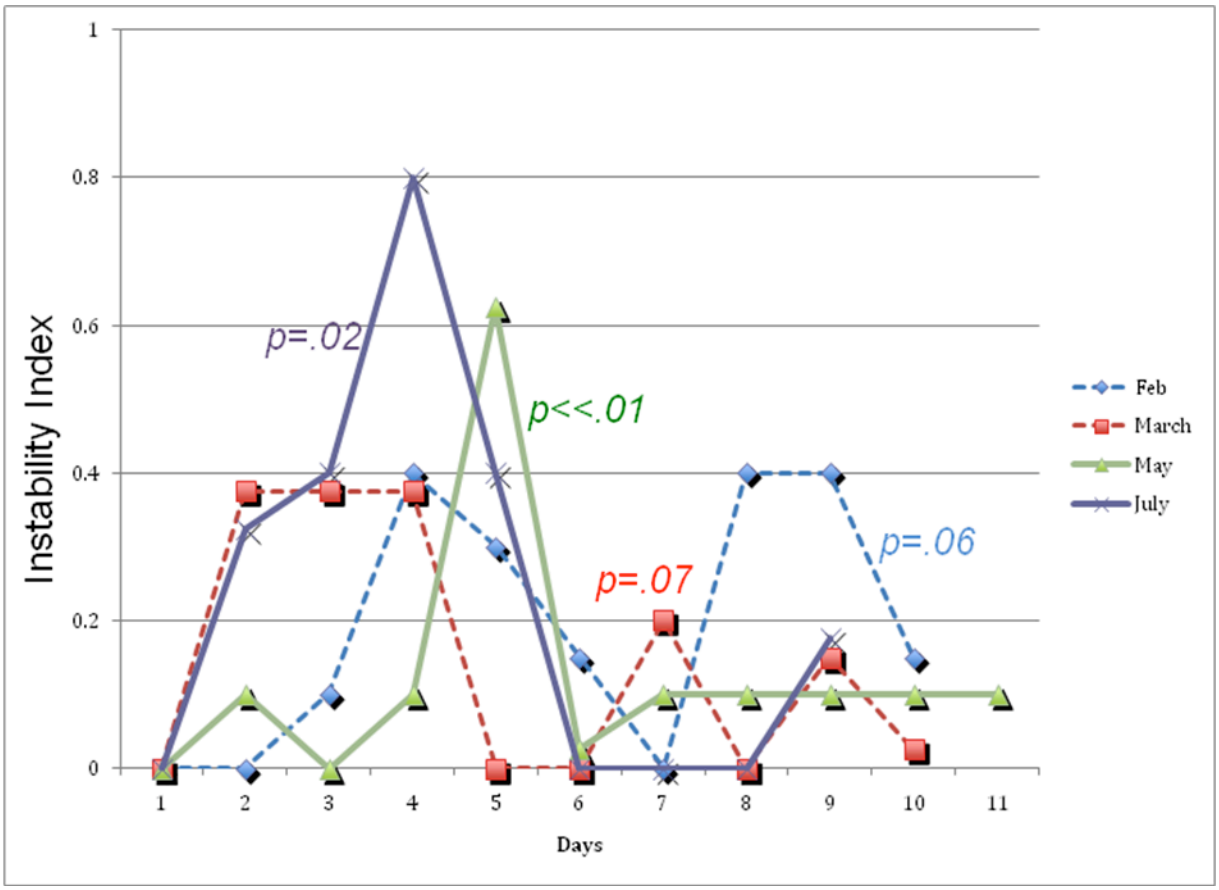


Figure 25. Daily changes in stability in the dominance hierarchy of four groups of fish. A permutation test was done for each group, and the p-values obtained are shown besides each group. Days 4 and 5 represent the highest instability. Note that February and March are spawning groups and May and July are non-spawning groups

Behavior Sequences: Spawning Season

The analysis for these two groups is presented in **Appendix A-1** and **Appendix A-2**. What follows is a summarized result of the findings. During spawning, nips accounted for 41% of all behaviors observed. In February, closer to the peak of the spawning season, color changes accounted for 34% of the behaviors observed. This number dropped to 2% in March. Frontal aggressions accounted for an average of 5% of observed behaviors. Aggressive chases accounted for 20% of the observed behaviors in February. This number went up in the March group: Aggressive chases accounted for 45% of all behaviors observed.

Spawning runs only occurred in the month of February, although in March some spawning-like behavior was also observed (data not shown). Despite this, many similarities exist between the months of March and February, when compared to the months of May and July.

Nips followed a zero-order Markov chain, in the spawning groups. There was no preferred response to a nip. However, most nips occurred by themselves: 53% of the nips were not followed by any behavior within 60 seconds of occurring.

During spawning, chasing seemed to behave as a first-order Markov chain. In February, at peak spawning, once a chase occurred, 34% of them followed with another chase (Chase→Chase: 34%). In March, this sequence did not follow first-order dynamics and no significant response to chases was observed in this month. No second-order Markov chain could be found for either month. Similar to nips, on average 44% of chases also occurred without any behavior following it.

Frontal aggressions followed a first-order Markov chain model. In February, the preferred response to a frontal aggression was a color change (HH→CC: 39%). However, in March, the preferred response was a nip (HH→OM: 22%). In both groups, the average percent of frontal aggressions that transitioned into a chase was 19% (HH→Chase: 19%). In March, the number of HH→HH transitions was higher than expected by chance, but it was still relatively low (HH→HH: 7%). However, the number of frontal aggression that occurred without any subsequent behavior was very high, 50%, when compared to February, in which only 33% of the recorded frontal aggressions occurred by themselves.

The response to a color change also differed between February and March. In February, the preferred response to a color change was another color change (CC→CC: 37%). The preferred response in March was a chase (CC→Chase: 36%). In both groups, some of the color changes were followed by a nip (CC→OM: 13%). Also on average, 45% of the color change events occurred with no behavior event occurring within the following 60 seconds.

Behaviors that were not preceded by any behavior accounted for many of the behavior events during these months, especially in February (February NA→OM: 71%; March NA→OM: 53%). Color changes were rarely preceded by an inactivity period (NA→CC: 35%). On average, chases occurred relatively frequently after periods of inactivity (Chase←NA: 43%). Frontal aggressions occurred usually as the first aggression in a behavior chain, or they occurred alone (NA→HH: 57%; NA→HH→NA: 39%).

Behavior Sequences: Quiescent groups.

The analysis for these two groups is presented in **Appendix A-3** and **Appendix A-4**. What follows is a summarized result of the findings. Behavior sequences were analyzed using zero order, first order and second order Markov Chain modeling with an inactivity threshold of 60 seconds. Behaviors were analyzed at the tank-level and not at the individual level. Thus, the analysis reflects not just the response of the attacked fish, but of every fish present in the arena. Note that for color changes and frontal aggression, the interpretation of the results of this analysis should consider the subjectivity of such measures. Color changes are hard to measure by simple video observations, and deciding who wins a "frontal aggression" is subject to observer bias.

During quiescent, the two most frequent behaviors, each representing on average about 44% of logged behaviors, were nips and chases. Nips are referred here in most figures as OM or Open-Mouth, due to the fact that often the animal is observed opening its mouth widely as it approaches the other fish. Nips were usually followed by another nip (OM→OM: 36%). If such a sequence occurred, the probability of another nip occurring in the tank was even higher (OM→OM when preceded by an OM→OM: 58%), which formed a second-order Markov Chain. On average, a nip was preceded by any other behavior (NA=No Activity) 38% of the time (NA→OM: 38%). Chase events were followed by more chase events (Chase→Chase: 37%). Similar to nips, when a chase followed another chase event, the probability of a subsequent chase occurring was very high (Chase→Chase when preceded by a Chase→Chase: 64%). It was not rare for chases to not follow any other behaviors (NA→Chase: 37%).

Frontal aggression (5%) and color change (8%) were not as common in the quiescent groups. Frontal (HH) and color change (CC) behaviors did not have a significant tendency to form first or second-order Markov Chains. Frontal aggressions were preceded by no activity 27% of the time, while color changes occurred with a period of inactivity before it, in 22% of the observed events (NA→HH: 27%; NA→CC: 22%).

The probability of a frontal aggression following a nip was very low, and less than expected by random chance (OM→HH: 5%). In the tetrad observed in July, a nip event had a small but significant chance of precipitating a color change event in the group (OM→CC: 12%). Thus, it can be inferred that during summer, the preferred response to a nip is another nip. These results can also be expressed by saying that nips usually occur in clusters.

Chases also followed first-order and second-order Markov chain dynamics. A chase was usually followed by another chase, and in the month of July the number of chases followed by a nip were less than expected by random chance (Chase→OM: 5%). Thus, the preferred response to a chase was also another chase.

Frontal aggressions were followed by chases (HH→Chase: 26%) or nips (HH→OM: 33%). The observed proportions of frontal aggressions that followed a frontal aggression were very low (HH→HH: 9%). A second-order Markov chain could not be fitted into this behavior sequence. The chance of a HH→HH→HH chain was very low, similar to the expected probability by chance. In July, some of the fish responded to frontal aggressions with a color change (HH→CC: 15%). Although very low, this calculated probability was higher than the expected value based on a zero-order Markov chain. Thus, the preferred response to a frontal aggression was a chase or a nip, except in July when some animals also responded with a color change.

Color changing behavior was difficult to observe and assigning an exact start or end time was very hard. Furthermore, the start and end of a color change are not analyzed separately. Indeed, color changing behavior and its associated transitions are subject to more observer bias than any other behavior analyzed here. No preferred response occurred, but it seemed that frontal aggression was avoided after a color change (CC→HH: 5%; CC→OM: 23%; CC→CC: 22%; CC→Chase: 23%; CC→NA: 26%).

Clusters

A cluster of behavior is defined as a group of behaviors that occur with no significant time lapse between behaviors. The period between clusters is a period of no activity or low activity. To form a cluster, behaviors had to occur within no more than 60

seconds of each other, and the cluster had to be at least three behaviors long. Behaviors could occur outside or within a cluster. A behavior chain is the same as a cluster. I use the term cluster when referring to the analysis of the behaviors that occur contiguously versus behaviors that occur in temporal isolation (as defined by the interval between clusters, which is 60 seconds here). Also behavior clusters represent a group of behaviors that occurred one after the other, regardless of the originator of the behavior and its relation to the originator of the previous or following behavior (e.g. the first behavior corresponds to the attacker, the second one is in retaliation of the first one and its originated by the attacked fish vs. the second behavior corresponds to another pair of fish not involved in the first behavior).

The analysis showed that most of the behaviors in the quiescent groups occurred within clusters (May: 74%; July: 50%). In the spawning groups, less than half of the behaviors occurred within clusters (February=39%; March=44%).

Position of Behaviors in Chains

Behavior chains occur when two or more behaviors occur one after the other. As defined here, an interval of 60 seconds or less can occur between the two behaviors. Along this chain, a specific behavior can be at the beginning, end, or middle of it. As noted above, a behavior can also occur without any other behaviors occurring around its temporal vicinity. Which behaviors begin or close a chain? Because there is very little that we know about seabream behavior, answering this simple question would extend our knowledge about the behavior mechanisms behind escalation, retaliation, and other agonistic events. This analysis is also useful to understand the meaning of some of the more enigmatic behaviors, such as frontal aggression and color change. The result of this

simple analysis is shown in **Figure 26**. It shows that nips and frontal aggressions were most likely to be at the beginning rather than at the end of a sequence. It also shows that color changes and chases were most likely to be at the end rather than at the beginning of a sequence.

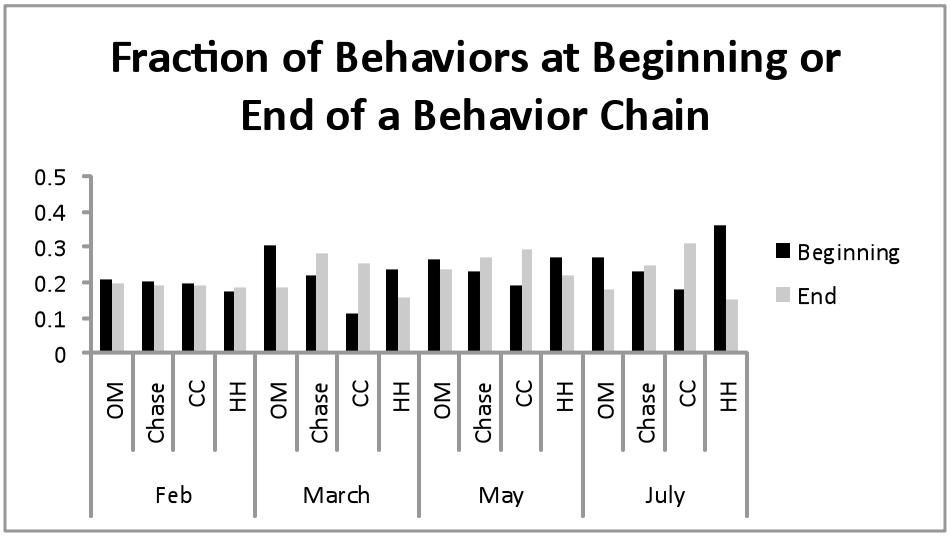


Figure 26. Fraction of behaviors at the beginning or at the end of a chain. Behaviors that occur by themselves or inside a chain are not shown for simplicity. Nips (OM) and frontal aggressions (HH) are usually in the beginning or middle of a chain, while Chases and color changes (CC) are occur at the end.

Discussion

Chases and nips are the two most common aggressive behaviors observed in seabream, together they account for an average of 80% of observed behaviors. The remaining 20% of behaviors counted are either frontal aggressions or color changes, suggesting that these behaviors play minor roles in establishing dominance or that they represent a significant escalation of aggression. There was no difference among the four groups in the occurrence of nips. When I applied a simple Markov-chain model to these behaviors, found that both nips and chases occurred as second-order Markov-chain processes during the period around which gonad involution takes place. In the spawning

groups, nips followed a zero-order process, while chases followed a first-order Markov chain at least in February, at the peak of spawning.

Nips and frontal aggressions were more likely to be at the beginning of a behavior chain than chases or color changes. Conversely, chases and color changes were more likely to be at the end of a behavior chain. There were no consistent differences among the spawning and sex changing fish in this aspect, suggesting that only the intensity of aggression changes during sex change season. Regardless of gonadal status, fish usually start an aggressive bout by nipping or performing a frontal aggression. These aggressive behaviors then escalate to chases and can culminate in a color change or end with a chase. It is possible that frontal aggression represent a way for a fish to challenge the dominance of another fish, and that nips represent the form of aggression used by fish to reinforce their dominance.

Markov chain modeling provides information about how the current state of a process is affected by its previous state. Recall that these analyzes were not performed at the level of the individual but at the level of the group. Thus, the fact that nipping and chasing follow a second order Markov-chain process only during the summer sex changing months suggests that aggressive behaviors have a domino effect: any aggressive act, triggers more aggressive acts in the group. When a fish is attacked, the state of the group changes, and the probability of another aggression occurring within the next minute is increased. The aggressor either attacks again or it is attacked. Also in some instances, an attack triggers observing fish, or audiences, to attack another member of the group. Such disturbances in the dominance structure can lead to rank changes. However, it is more likely that they are part of the group dynamics that lead to reinforcement of

dominance ranks. Since I did not analyze data at the individual level, the presence of a single despotic individual could have produced the observed result. Despotic individuals are those that can dominate all members of the group. Despotic individuals are not only the most dominant individuals, they are the only dominant individuals. Hence, in a despotic society, only one individual is dominant while the rest are all subordinates to it. A despot can attack more than one fish, thus accounting for the multiple aggression events occurring so close to each other. However, the result of the analysis of dominance rank in these groups does not seem to support the existence of a despotic dominance structure: More than one individual seemed to dominate in each of the four groups.

Aggressive chases were less frequent in the February (Spawning) group. This was also the only month were true spawning chases were consistently observed. Perhaps this was due to observer's bias, which occurred because of how difficult it is to differentiate a spawning chase from an aggressive chase. It is also likely that this decrease in aggressive chases was due to an overall decrease in aggression that resulted from the fish being more motivated to spawn than to engage in territorial disputes. The results of the dominance rank analysis, stability calculations, and the frequency of overall aggression for each of the four groups is consistent with such a motivational shift.

Another aspect I measured was the percent of clustered behaviors. This is the number of behaviors that occur within 60 seconds of each other over the total number of behaviors analyzed (i.e. total of nips, chases, frontal, and color changes). Thus, clusters represent bouts of aggression. The percent of clustered behaviors was higher in May and July (gonad regression), indicating that bouts of aggression are more common during this time. Average size of each aggression cluster was also higher for these months, although

perhaps the differences were not significant (data not shown). Considering that the results of Markov-chain models also suggested increased aggression intensity during these months, the results of these analyzes consistently suggest that during gonad regression, the fish are more aggressive, and more likely to defend their dominance rank. Conversely, during spawning aggression events can be sporadic and isolated, and the fish are less aggressive.

The analysis of these behavior sequences suggests that the gonad cycle has a direct effect on the aggressive behavior of the Gilthead seabream. This fact however does not answer any of the key questions I posited in the beginning of this chapter about the mechanisms by which sex change might be affected by behavior. The modeling of the behavior sequences suggests that the increased in aggression intensity coincides with gonad regression. Because gonad regression occurs within the nadir of gonadal steroid production (see **Chapter 3**), it is clear that such an effect is not simply the result of the action of the gonadal steroids *per se*, but rather that perhaps other hormones that control gonad maturation and regression might be involved in the increased aggression. Although it seems that gonad steroids do not need to exist at detectable levels during the non-breeding season in order to stimulate aggression in birds (Review: Soma et al., 2008). However, in the group of fish that I have analyzed here, I did not measure blood steroids. For such a study, refer to **Chapter 4**, where I discuss the results of another experiment in which both testosterone and estradiol were measured in plasma of fish in which behavioral observations similar to the ones described here were performed.

Because male and female fish have a different gonad steroid profile (see **Chapter 3**), a simple way to address this problem is to look at the difference in the expression of

aggressive behavior among the two sexes and the same individuals before and after gonad regression. Are female more aggressive than males?

I expected that, just like clownfish (Godwin, 1994) and wrasses (Warren and Swearer, 1991), the terminal sex (for seabream, the female) would be more aggressive**.** This hypothesis was supported by the first experiment I performed, the dyadic encounters between size-matched females and males. Furthermore, sex-matched pairs seem to follow the traditional rule that a big fish dominates a smaller fish. However, in the tetrads, where females were also bigger than the males, the expected outcome did not occur. Here, the smaller male seemed to dominate over the larger females, especially during the sex changing period. Also, mature males were more aggressive than females in the two spawning groups observed . The difference was significant in February but not in March. In the May group, the frequency of intersexual and intrasexual aggression was equal, but in the other three groups, intrasexual aggression was more frequent than intersexual aggression. Why is this finding significant?

Previous work by Wong (2006), a former member of the Zohar lab, has shown a significant increase in P_{450} aromatase levels in gonad tissue of seabream during the month of May, June and July (Wong et al. 2006). Because aromatase may be involved in gonad remodeling and sex change, and because we have observed a similar increase in brain GnRH-2 and AVT mRNA transcripts around the same period (**Chapter 3**), we surmise that by June the gonadal sex has already been established. Therefore, it is not surprising that during May, aggression towards females increased. The possibility also exists that the window for sex determination, what we have also termed the sensitive period, is much narrower than we had anticipated. Thus, after May the sex of the animal could

have also been determined. It is also possible that these hormones peak not because sex has been decided, but because sex will be decided soon. In preparation for this protracted sex change, the animal might develop certain regions of the brain which control aggression. Meanwhile, the convergence of gonadal and brain events might occur simply because the gonad itself is also preparing for sex change. This proposed development cycle implies that the gonad and brain can be primed for sex change before sex change *per se* takes place. This is only possible because in seabream sex change is a protracted process, taking at least 3 months. Thus, in the sparid gonad sex change begins before the outcome has been decided. Only by using environmental cues obtained by social interaction, do the specific pathways activate to promote sex change.

Why were females in the tetrads less aggressive than males? An alternative explanation is that, because males in this experiment where younger than the females, younger individuals are more aggressive than older individuals. Younger fish may have higher levels of 11-KT, a hormone we did not measure, which has been shown to be higher in juvenile gag (Heppel 2005). Indeed, a surge in steroidal hormones accompanies the maturation and silvering of juvenile eels (van Ginnecken at al. 2007) and 11-kt may be one of the most crucial of these hormones (Rohr et al. 2001). Lastly, another alternative explanation is that perhaps the large size difference between females and males caused the females to consider the smaller males as non-threatening. Therefore, they did not need to fight them off. Using a mathematical model, Hock and Huber (2007) have found that in pairs of animals in asymmetric versus symmetric socially embedded dyads, closely matched opponents were less effective at promoting a social hierarchy within the group than asymmetric contestants. Most aggression occurred between males

and between females, except in the month of May, when both inter- and intra- sexual aggression occurred with the same frequency. Thus, this alternative explanation is at least partially supported by our data. The fact that the month of May was an exception, stills suggest that around this month, dominance behaviors between the females and males serve to suppress or initiate sex change. Another explanation for the observed increase in intrasexual aggression during spawning is that during this period mate-competition is emphasized over any other aspect of dominance. We observed a higher frequency of spawning runs during February (not shown). Male aggression increased during this month, and intrasexual aggression was higher or almost as high as intersexual aggression. Thus, on the surface, there seems to be some evidence of mate competition. If this explanation is correct, then perhaps intersexual aggression does not increase during sex change but simply decreases during spawning and is overcome by intrasexual aggression during spawning. The fact that there was an increase in aggressive behavior during the quiescent month of May and not during the spawning month of February, does not support this explanation. Thus, close examination of the data suggests that absence of mate competition does not cause an artifact increase in intersexual aggression during the quiescent season.

We expected that the formation and maintenance of hierarchies would depend on the gonad stage. Seabream have a complex gonad cycle that has been well characterized (Zohar et al. 1978; Zohar, 1984). Since the gonad is an important site for the biosynthesis of steroids, we expected that the presence or absence of a developed gonad would influence the behavior of the fish. Furthermore, because during the spawning season the animals must come together to spawn in small groups, we expected the

formation of weak and unstable hierarchies during this period. The results shown here suggest that both quiescent and spawning groups have unstable and relatively linear hierarchies during the first five days of interaction. However, after the hierarchy stabilizes, which I interpreted as the days following peak instability, the hierarchies only become linear in the quiescent groups. Spawning fish in the February group did not reach stability even after day 8, but the other three groups seemed to have stabilized afterwards. Still, the Instability permutation test that I developed (**Chapter 2**), suggests that the hierarchies on average are more stable in the quiescent groups than in the spawning groups. Thus, the above-stated hypothesis was supported by the lack of stability in the spawning groups and the formation of linear and stable hierarchies among the tetrad members during gonad quiescence.

The fact that the first five days of interactions were marked by increased instability suggests that the Gilthead seabream is sensitive to changes in the composition of its social groups. When a tetrad was initiated by mixing individuals from different tanks (group fission and fusion), the individuals took time to re-establish dominance relationships. Such sensitivity to social perturbations may predispose seabream to manipulations of the social environment that affect the hierarchy, and consequently may influence sex change, and may also be the key to understanding why seabream populations in captivity tend to develop an overabundance of females.

In conclusion, seabream are more aggressive and form relatively stable linear hierarchies during the prolonged period that precedes sex change (quiescence). During the spawning period, this species shows decreased aggressive behavior and groups of fish do not show formation of strong linear hierarchies. The limited data set that we have

analyzed here also suggests that during the month of May, seabream have a significant increase in the frequency of aggressive behavior. It should be noted that due to the small number of animals per group and the small number of groups per gonad stage, it is difficult to conclude beyond doubt that a relationship exist between gonadal cycle and aggressive behavior. However, the experimental design allows me to draw some general conclusions about spawning vs. quiescence, and the fact that such differences exist in other species (e.g. Trainor et al., 2007; Soma et al., 2008) makes it easier to reach these conclusions. Still, care should be taken when interpreting these results. For example, the higher activity in the May tetrad (Quiescent gonads) could have been due to the abnormal behavior of only one individual. Until an experiment is done, in which several replicate groups are observed in different tanks but at the same time, the risk of a single individual behaving abnormally different, could alter the results. Indeed, I attempted to do such an experiment later (see **Chapter 4**), but with a different goal in mind.

Regardless, the differences between spawning and quiescent groups in the experiment presented here are statistically significant, offering the only line of evidence that in the Gilthead seabream, behavior is affected by gonad stage in a manner consistent with Sex Allocation Theory. Furthermore, this study has provided the only analysis of behavior sequences in seabream and can serve as the basis for other studies using this fish species as a model. This study has also provided evidence that the basic mechanisms for sex change to be influenced by behavior exist in the Gilthead seabream: fish form linear and relatively stable hierarchies, social perturbations such as group fusion destabilize the groups, and the animals seem to have a sex-biased dominance hierarchy. Since analysis of the gonad tissue using molecular biology tools suggested that males are committed to

sex change around the month of July (Wong and Zohar 2004), and since this data also correlates well with similar analysis of brain tissue (**Chapter 3**), I believe that both behavioral and gonadal events may converge around this period to establish the outcome of sex change.

Thus, both behavioral and molecular data suggest that during the quiescent period, important changes occur in the behavior, gonad histology, and brain neurochemistry of the fish. Further experiments are needed to understand the causal relationship between this behavioral and neurendocrine correlates (see **Chapter 3** and **4**). The work that I have presented here lays the foundation for further research into the interactions between neuroendocrine factors and behavior in this species.

FEBRUARY							
==========							
= **Sequence Analysis** =							
= **AquaObserver vA12** =							
==========							
Bin Resolu	450						
Forward P	Probability of FROM going to TO						
From	**To**	**Forward I**	**Background F**	**Transitions (TTL)**	**Behavior (TTL)**	**Ant. Transition**	**Ant. Transition P**
N.A	N.A	15	.	15	1		
N.A	CC	67	.	67	196		
N.A	OM	163	.	163	229		
N.A	CHASE	44	.	44	113		
N.A	HH	18	.	18	33		
CC	N.A	0.44	0.34	87	1		
CC	CC	0.37	0.34	72	196	CC->CC	0.33
CC	OM	0.08	0.34	16	229	CC->CC	0.56
CC	CHASE	0.05	0.34	10	113	CC->CC	0.8
CC	HH	0.03	0.34	6	33	CC->CC	0.83
OM	N.A	0.59	0.4	134	1		
OM	CC	0.12	0.4	28	196	CC->OM	0.36
OM	OM	0.16	0.4	36	229	OM->OM	0.53
OM	CHASE	0.06	0.4	14	113	OM->OM	0.43
OM	HH	0.04	0.4	9	33	OM->OM	0.44
CHASE	N.A	0.4	0.2	45	1		
CHASE	CC	0.14	0.2	16	196	CHASE->CHASE	0.81
CHASE	OM	0.11	0.2	12	229	CHASE->CHASE	0.5
CHASE	CHASE	0.34	0.2	38	113	CHASE->CHASE	0.42
CHASE	HH	.	0.2	0	33		
HH	N.A	0.33	0.06	11	1		
HH	CC	0.39	0.06	13	196	OM->HH	0.46
HH	OM	0.06	0.06	2	229	HH->HH	0.5
HH	CHASE	0.21	0.06	7	113	OM->HH	0.57
HH	HH	.	0.06	0	33		
Min. Clust	3						
Inactivity I	900						
Clusters:	38						
TTL Behav	220						
TTL Behav	571						
Percent in	0.385289						

Appendix A-1. ***Transition probability matrix for February Tetrads. Note anterior transitions (2nd Order Markov chain or Lap-2) on the right hand side. Percent of clustered behaviors is 39%.***

MARCH							
==========							
= **Sequence Analysis** =							
= **AquaObserver vA12** =							
==========							
Bin Resluti	450						
Forward P	Probability of FROM going to TO						
From	**To**	**Forward P**	**Background F**	**Transitions (TTL)**	**Behavior (TTL)**	**Ant. Transition**	**Ant. Transition P**
N.A	N.A	15	.	15	1		
N.A	OM	141	.	141	267		
N.A	CHASE	132	.	132	285		
N.A	HH	42	.	42	72		
N.A	CC	4	.	4	11		
OM	N.A	0.47	0.42	126	1		
OM	OM	0.21	0.42	57	267	OM->OM	0.53
OM	CHASE	0.22	0.42	58	285	OM->OM	0.47
OM	HH	0.06	0.42	16	72	OM->OM	0.63
OM	CC	0.01	0.42	2	11	CHASE->OM	1
CHASE	N.A	0.48	0.45	137	1		
CHASE	OM	0.18	0.45	51	267	CHASE->CHASE	0.55
CHASE	CHASE	0.28	0.45	79	285	CHASE->CHASE	0.54
CHASE	HH	0.03	0.45	9	72	CHASE->CHASE	0.44
CHASE	CC	0.01	0.45	4	11	CHASE->CHASE	0.75
HH	N.A	0.5	0.11	36	1		
HH	OM	0.22	0.11	16	267	OM->HH	0.44
HH	CHASE	0.17	0.11	12	285	CHASE->HH	0.5
HH	HH	0.07	0.11	5	72	CHASE->HH	0.4
HH	CC	0.01	0.11	1	11	OM->HH	1
CC	N.A	0.45	0.02	5	1		
CC	OM	0.18	0.02	2	267	CHASE->CC	0.5
CC	CHASE	0.36	0.02	4	285	CHASE->CC	0.5
CC	HH	.	0.02	0	72		
CC	CC	.	0.02	0	11		
Min. Clust	3						
Inactivity I	900						
Clusters:	37						
TTL Behav	282						
TTL Behav	635						
Percent in	0.444094						

Appendix A-2. ***Transition probability matrix for March Tetrads. Note anterior transitions (2nd Order Markov chain or Lap-2) on the right hand side. Percent of clustered behaviors is 44%.***

MAY							
==========							
= **Sequence Analysis** =							
= **AquaObserver vA12** =							
==========							
Bin Resolu	450						
Back P:	Probability of TO coming from FROM						
From	**To**	**Forward P**	**Backgrou**	**Transitions (T**	**Behavior (TTL)**	**Ant. Transition**	**Ant. Transition I**
N.A	N.A	3.5	.	7	2		
N.A	CHASE	225.5	.	451	1638		
N.A	HH	31	.	62	274		
N.A	OM	309	.	618	2156		
N.A	CC	11	.	22	81		
CHASE	N.A	0.28	0.39	456	2		
CHASE	CHASE	0.38	0.39	615	1638	CHASE->CHASE	0.56
CHASE	HH	0.05	0.39	74	274	CHASE->CHASE	0.45
CHASE	OM	0.29	0.39	479	2156	OM->CHASE	0.45
CHASE	CC	0.01	0.39	11	81	CHASE->CHASE	0.64
HH	N.A	0.25	0.07	68	2		
HH	CHASE	0.28	0.07	76	1638	OM->HH	0.47
HH	HH	0.1	0.07	27	274	CHASE->HH	0.41
HH	OM	0.35	0.07	96	2156	OM->HH	0.51
HH	CC	0.03	0.07	7	81	CHASE->HH	0.57
OM	N.A	0.28	0.52	602	2		
OM	CHASE	0.22	0.52	474	1638	OM->OM	0.53
OM	HH	0.05	0.52	107	274	OM->OM	0.6
OM	OM	0.44	0.52	945	2156	OM->OM	0.63
OM	CC	0.01	0.52	25	81	OM->OM	0.64
CC	N.A	0.26	0.02	21	2		
CC	CHASE	0.27	0.02	22	1638	CHASE->CC	0.36
CC	HH	0.05	0.02	4	274	HH->CC	0.75
CC	OM	0.22	0.02	18	2156	OM->CC	0.56
CC	CC	0.2	0.02	16	81	OM->CC	0.44
Min. Clust	3						
Inactivity I	900						
Clusters:	215						
TTL Behav	3067						
TTL Behav	4150						
Percent in	0.739036						

Appendix A-3. ***Transition probability matrix for May Tetrads. Note anterior transitions (2nd Order Markov chain or Lap-2) on the right hand side. Percent of clustered behaviors is 74%.***

JULY							
==========							
= **Sequence Analysis** =							
= **AquaObserver vA12** =							
==========							
Bin Resolution:	450						
From	**To**	**Forward P**	**Backgrou**	**Transitions (T**	**Behavior (TTL)**	**Ant. Transition**	**Ant. Transition I**
N.A	N.A	4	0.01	8	2		
N.A	OM	27	0.01	54	115		
N.A	CHASE	35.5	0.01	71	158		
N.A	CC	3.5	0.01	7	45		
N.A	HH	2	0.01	4	13		
OM	N.A	0.32	0.35	37	2		
OM	OM	0.31	0.35	36	115	OM->OM	0.53
OM	CHASE	0.17	0.35	19	158	OM->OM	0.53
OM	CC	0.12	0.35	14	45	OM->OM	0.43
OM	HH	0.05	0.35	6	13	OM->OM	0.67
CHASE	N.A	0.49	0.48	77	2		
CHASE	OM	0.06	0.48	10	115	CHASE->CHASE	0.7
CHASE	CHASE	0.36	0.48	57	158	CHASE->CHASE	0.72
CHASE	CC	0.07	0.48	11	45	CHASE->CHASE	0.45
CHASE	HH	.	0.48	0	13		
CC	N.A	0.27	0.14	12	2	CC->CC	0.08
CC	OM	0.24	0.14	11	115	CC->CC	0.55
CC	CHASE	0.18	0.14	8	158	CC->CC	0.5
CC	CC	0.24	0.14	11	45	OM->CC	0.36
CC	HH	0.04	0.14	2	13	OM->CC	1
HH	N.A	0.23	0.04	3	2		
HH	OM	0.31	0.04	4	115	OM->HH	0.5
HH	CHASE	0.23	0.04	3	158	OM->HH	0.67
HH	CC	0.15	0.04	2	45	N.A->HH	0.5
HH	HH	0.08	0.04	1	13	OM->HH	1
Min. Cluster Size:	3						
Inactivity Intracluster Threshold:	900						
Clusters:	19						
TTL Behaviors in Clusters:	165	8.684210526					
TTL Behaviors Analyzed:	332						
Percent in Clusters:	0.496988						

Appendix A-4. ***Transition probability matrix for July Tetrads. Note anterior transitions (2nd Order Markov chain or Lap-2) on the right hand side. Percent of clustered behaviors is 50%.***

Chapter 3: AVT and GnRH expression in the brain of seabream during the gonad cycle.

Introduction

The relationship between dominance rank and sex change has been explored in clownfish (Fricke and Fricke, 1977), and wrasses (Semsar and Godwin, 2003). There is evidence in several other hermaphrodite species that social environment influences sex change (e.g. Lorenzi and Grober, 2006; Carruth, 2000). In the Gilthead seabream, it seems that sex change outcome is also influenced by the social context (Happer and Zohar, 1988). The exact mechanism is unknown, although Lorenzi and Grober (2006) have proposed that direct contact is needed between fishes to allow sex change suppression in the goby. A similar argument has been made about clownfish, because removal of the female triggers sex change and the female seems to constantly show dominance over the male with both nipping behavior and aggressive displays (Godwin, 1994). How sex changers interpret their social environment is not know, although it is evident that the Hypothalamus-Pituitary-Gonadal (HPG) axis plays the key role in translating these signals in hermaphroditic vertebrates (Review: Godwin et al., 2003).

In wrasses, behavioral sex change precedes gonadal sex change and can occur in the complete absence of gonads (Godwin et al., 1996). This implies that the brain itself controls sex change, and that gonadal steroids do not play a central role in suppressing or enabling this process. Indeed, Foran and Bass (1999), proposed two hypothalamic neuropeptides as the putative axes for sexual plasticity in teleost fish: Gonadotropin Releasing Hormone (GnRH) and Arginine Vasotocin (AVT). Because they are central to the sexual plasticity of the teleost brain, these hormones could be the target substrate of

other neuroendocrine factors acting as transducers of social environment. Indeed, such factors themselves could control or enable sex change.

In this chapter, I investigate the expression of AVT and GnRHs in the brain of the Gilthead seabream, during different stages of the gonadal cycle. Elucidating the seasonal changes in the concentration of these neuroendocrine factors will provide the basis for understanding the question of when (and how), such endocrine factors can be influenced by the social environment. The seasonal profile of AVT and the GnRH system will elucidate more than just the length and timing of sex change, but it might help identify specific events in the development of the gonad and of the process of behavioral sex change. I hypothesize that AVT, and GnRH-2, which are two neuropeptides associated with controlling sexual and territorial behavior, will be differentially expressed among males and females because they are both associated with territorial and dominance behavior in fish. Dominance behavior and control of territory can be linked to sex change because sex allocation predicts that control of resources, investment of energy in the control of resources, and dominance, are sex-biased.

The gonadal cycle of seabream and other protandrous species suggests that there is a "window" of sensitivity to social and environmental cues. This implies a putative point-of-no-return for the ambisexual gonad, after which sex is no longer mutable until the next round of sex reversal or until terminal sex has been selected by such cues. Zohar et al. (1986) postulated that a sex reversal window probably existed in ambisexual seabream during the summer months, when the bipotential gonad can still develop into either a testes or an ovary. Wong (2003) was able to show that if such a window existed in seabream, it occurred during the months of June and July. In black porgy, a closely

related protandrous hermaphrodite which also has winter spawning, Lee et al. (2002), surmised the existence of an even earlier but protracted window, beginning in spring, and which extends to just a few months before spawning.

In this chapter, I would like to address this question by comparing the endocrine cycle to the data collected and analyzed by Wong (2003) on seabream gonad development. I also analyzed subjective color measurements of seabream prespawning and spawning coloration. When does the putative sex reversal "window" open and how long does it take for a fish to commit to one sex or the other? While the experiment was designed to characterize the seasonal profile of AVT and GnRH's, the data collected could also be used to provide a partial answer to this question. The question can be framed within the following hypothesis: Similar to black porgy (Lee et al., 2002), a related species with protandrous hermaphroditism and a similar gonad cycle, the sex reversal window in seabream extends from summer to early spring.

Another hypothesis that could be tested, even more directly, is that peri-spawning and spawning coloration are sexually dimorphic and that, consistent with sex allocation theory (Ghilsein, 1969) and the life history of seabream, this coloration is more intense in females. In clownfish, wrasses and many other reef fish, the terminal sex is the most colorful because this fish are more aggressive, and they have more energy invested in the shortest period of time, making advertisement of their reproductive status very important. Thus, even when the experiment was designed to simply show the AVT and GnRH hormonal profile, and how it relates to other reproductive parameters, it also provided the opportunity to test these basic questions about seabream life history and reproductive behavior, which have never been tested.

The GnRH System

GnRH is the central regulator of gonad development (Porterfield, 1996). In vertebrates, GnRH coordinates the development of ovaries or testes, and the maturation of the gametes, by inducing the release of the two pituitary gonadotropins: Lutenizing Hormone (LH) and Follicle Stimulating Hormone (FSH). Both of these events are highly regulated, and GnRH serves as the central mediator of that regulation (Review: Zohar et al., 2009). Perhaps because of the requirement to rigorously coordinate and time these processes, vertebrates have evolved several isoforms of GnRH. Each isoform of GnRH seems to coordinate different aspects of reproduction (Weltzien et al., 2004). While most species studied have at least two GnRH isoforms, amphibians and many teleost species, including the Gilthead seabream, have a third form (Chen and Fernald, 2008; Pham et al., 2006; Mohamed et al., 2005; Powell et al., 1994). Each of these isoforms, is present in specific regions of the brain (Gothif et al. 1995). The specificity of the location of these different GnRHs, suggest that they also serve very specific functions in the brain. Moreover, GnRH multiplicity provides the GnRH axis with a wide range of putative modulators, because each of these isoforms is located in different regions of the brain where they are influenced by different neuroendocrine factors (Hoffman, 2006).

In perciform fish which have the 3-form GnRH system, such as the Gilthead seabream, GnRH-1 is expressed by cells in the hypothalamus which directly synapse pituitary gonadotropes and stimulate the pituitary to release LH and FSH. This so-called POA-GnRH, because it is found in the preoptic nucleus of the hypothalamus, is the endogenous releaser of gonadotropins (Review: Oka et al., 2009). In the gilthead seabream, this GnRH corresponds to GnRH-1 (Parhar, 2002; Powell et al., 1994). Social

stress down regulates GnRH-1 in tilapia (Ogawa et al., 2003). The existence of such a mechanism in tilapia, provides a potential explanation for the effect that social context has on reproductive status in many fish. GnRH-1influences gonad maturation (Hoffman, 2006); Thus, when GnRH-1 is downregulated (by social stress), the fish gonad maturation is affected. This relationship can provide a mechanism in which the effects of behavior (via social stress) can affect the speed of gonad maturation (via GnRH-1). In seabream, gonads mature asynchronously in the population, and it is believed that the rate of gonad maturation affects the outcome of sex change. The females that develop faster will reach the committal point before the ambisexual males, and through an as-yet unidentified mechanism, would "tell" those remaining uncommitted fish to stop changing sex. In this way, GnRH-1 seems to be a likely candidate to be the most important regulator of sex change.

GnRH-2 is ubiquitously expressed by cells in the midbrain. Zohar et al. (1995) posited that GnRH-2 is a general neurotransmitter throughout the brain. GnRH-2 does not seem to be involved in gonadal development or release of LH (Review: Zohar et al., 2009; Gault et al., 2003). However, there is ample evidence linking GnRH-2 to the control of reproductive behaviors (Hoskias et al., 2008; Kauffman and Rissman, 2004; Temple et al., 2003; White et al., 1995). Like GnRH-1, GnRH-2 is also down regulated by stress in tilapia (Ogawa et al., 2003), suggesting that reproductive behaviors can be directly affected by social context.

In perciform fish with 3 GnRH isoforms, the third form of GnRH, GnRH-3, is present almost exclusively in the Terminal Nerve and Olfactory Bulb (Amano et al.,

1991). This isoform is thought to modulate visual or olfactory information relevant to

	Vertebrate Vasopressin Family	
Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2	Argipressin (AVP, ADH)	Most mammals
Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH2	Lypressin (LVP)	Pigs, hippos, warthogs, some marsupials
Cys-Phe-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2	Phenypressin	Some marsupials
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH2	Vasotocin†	Non-mammals
	Vertebrate Oxytocin Family	
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2	Oxytocin (OXT)	Most mammals, ratfish
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Ile-Gly-NH2	Mesotocin	Most marsupials, all birds, reptiles, amphibians, lungfishes, coelacanths
Cys-Tyr-Ile-Gln-Ser-Cys-Pro-Ile-Gly-NH2	Seritocin	Frogs
Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-Gly-NH2	Isotocin	Bony fishes
Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Gln-Gly-NH2	Glumitocin	Skates
Cys-Tyr-Ile-Asn/Gln-Asn-Cys-Pro-Leu/Val-Gly-NH2	Various tocins	Sharks
	Invertebrate VP/OT Superfamily	
Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH2	Diuretic Hormone	Locust
Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly-NH2	Annetocin	Earthworm
Cys-Phe-Ile-Arg-Asn-Cys-Pro-Lys-Gly-NH2	Lys-Connopressin	Geography & imperial cone snail, pond snail, sea hare, leech
Cys-Ile-Ile-Arg-Asn-Cys-Pro-Arg-Gly-NH2	Arg-Connopressin	Striped cone snail
Cys-Tyr-Phe-Arg-Asn-Cys-Pro-Ile-Gly-NH2	Cephalotocin	Octopus
Cys-Phe-Trp-Thr-Ser-Cys-Pro-Ile-Gly-NH2	Octopressin	Octopus

†Vasotocin is the evolutionary progenitor of all the vertebrate neurohypophysial hormones. Vasotocin is only found in hagfish & lampreys.[9]

Figure 27. The peptide sequence of the AVP/Oxytocin Superfamily of Neuropeptides is highly conserved. (*From: http://en.wikipedia.org/wiki/Vasopressin*)

reproductive behavior (Zhang and Delay, 2007; Grens et al., 2005; Wielchmann and Wielchmann, 2001; Yamamoto and Kawashima, 1997). Ogawa et al. (2006) have proposed that GnRH-3 is in itself a modulator of behavior.

The AVT System

AVT, the second axis of sexual plasticity mentioned by Foran and Bass (1999), is a highly conserved nonapeptide. AVT belongs to a family of neuropeptides, which includes oxytocin, vasopressin, and many other related peptides (**Figure 27**). These peptides control affiliative and territorial behavior in most vertebrates. In mammals, vasopressin is also involved in the response to hypovolemia and stress, while oxytocin is involved in lactation (Porterfield, 1996). In lower vertebrates and invertebrates,

analogous peptide sequences have been identified (Liu and Ben-Jonathan, 1994). Interestingly, from the locust (Hexapoda: Insecta) to the vertebrates, these peptides seem to be involved in the control of water balance (**Figure 27**). A similar peptide sequence has also been identified in annelids (Oumi et al., 1994) and gastropods (Sawyerb et al., 1984).

Evidence from experiments in mammals, birds, reptiles and fish suggest that both AVT, and its mammalian analogue Arginine Vasopressin (AVP), are involved in the control of reproductive and social behavior (Review: Insel and Young, 2000). Thus, these peptides can control the expression of male and female behavior. Because hermaphrodite fish can change sex *ex ovum*, the hormones that control male and female behavior also serve as permissive factors enabling their extreme behavioral plasticity. In this context, AVT becomes an axis for sexual plasticity, because it both controls the expression of behavior as well as responds to changes in the social environment. This commutability, which is typical of many hormones, makes AVT an ideal target for exploring the correlation between the changes in the social environment (e.g., the removal of a dominant individual) and sex change. There is also evidence that AVT can trigger specific behavioral sequences, such as the spawning reflex in killifish (Pickford and Strecker, 1977). Indeed, AVT is involved in the control of reproductive behavior in many fish species (Review: Foran and Bass, 1999).

Gonadal Steroids

Steroids, whether gonadal or extra-gonadal in origin, can affect the AVT and GnRH axis. Furthermore, in fish dominance status can affect testosterone and 11-ketotestosterone levels (e.g. Cardwell et al. 1996). AVT expression in the brain has been

shown to be under the influence of gonadal steroids (Boyd, 1994). A gonad steroid feedback loop for the GnRH system has been found and has been extensively studied in fish (Review: Lee et al., 2008). Thus, it is important to measure those steroids produced by the gonads that may affect these axes. It is also important to measure gonad steroids because they can affect the behavior of the fish in ways that can also influence the outcome of sex change. Indeed, the GnRH axis, the AVT axis, the brain centers that control sexual and social behavior, and the ambisexual gonad itself, constitute a contiguous neuroendocrine network that ultimately, direct and control the development of the hermaphroditic fish gonad.

Pre-Spawning and Spawning Coloration

Both the common Spanish name, "dorada" (golden), and the scientific Latin name, *Sparus aurata* (*aurata*=gold), for the Gilthead seabream make a direct reference to the fact that this fish has a golden stripe in its "forehead", right above the eyes. However, this is not the only marking in this fish. Other body markings in Seabream seem to intensify as the spawning season approaches. A golden-orange band, vertical to the main axis of the fish, is present in this species year-round, but is intensified during the spawning season. A distinctively yellow line also appears in the underbelly of the fish, parallel to and in the region where the gonads are found. To my knowledge, no one has ever identified these as potentially sexually dimorphic characters, even when such markings should be presumed to be dimorphic (because they are usually seen during spawning).

Because the spawning grounds are considerably shallower than the open-waters in which seabream are found, orange and yellow markings are sufficient advertisement

signals for this species. These markings are probably enhanced during aggressive confrontations, when the fish change color. As noted in **Chapter 1**, it is not unusual for Seabream to change melanic color intensity during periods of territorial contests and prolonged bouts of aggression. If these melanic color changes occur during spawning season, the intensity of the spawning colors is exaggerated in the individuals that become dark (Thus, it is possible that melanic color changes play a different role during spawning). A darker fish will probably advertise its color markings better because of the effect known as contrast enhancement (an orange or yellow mark is more visible with a dark background than a grey-white background). This phenomena was easily observed when seabream were housed in the only tank in the facility that had a viewing window: often a single fish would be observed swimming in the same spot in the water column, in a "holding" position, while other fish passed by, breaking its position occasionally to chase other fish away, and always having an orange spot visible against its very dark melanic color.

If the intensity of these spots is a sexually dimorphic character, then it is also possible that such secondary characters are under the influence of gonadal steroids. Also, if the markings appear before spawning, they might serve a purpose different than spawning readiness and sex advertisement. In fact, color changes and color markings are potential signals that can translate social context. In this Chapter, these hypotheses about seabream spawning coloration are also explored.

METHODS

Experimental Animal Holding and Sample Collection

Approximately, 84 two-year old male Gilthead seabream were held in four, 2.0 m^3 tanks

in COMB's Aquaculture Research Center and exposed to simulated natural photoperiod conditions, consisting of 16-H of daylight in the summer, and 8-H of daylight in the winter. To simulate the natural seasonal fluctuations in water temperatures, tank water also followed a similar pattern, with temperatures ranging from 15°C in the winter, and gradually increasing to 23°C for the summer period. These conditions simulate the natural environment that this fish might encounter in the Mediterranean Sea. Fish were fed on commercial "seabream M" diet (EWOS Canada Ltd, BC, Canada) at 1.5 to 2 % of body weight. Each month, 8 ambisexual or 16 fish (8 males and 8 females) were anesthetized in 200 ppm 2-phenoxyethanol. A total of 96 fish were euthanized for the experiment. A 1.0 ml blood sample was drawn from each fish's caudal peduncle. The brain of each animal was carefully extracted and placed in dry ice for later RNA extraction and analysis.

Primer Name	Sequence	Description
AVT21F	CCTCGCAGCAGGACATACAG	*Forward Primer for Reading/Cloning ORF*
AVT521R	GGGCATTTTATTGGTTCAGGG	*Forward Primer for Reading/Cloning ORF*
AVT_287F	CAGCTCACTGTGTGGAGGAGGAG	*Forward Primer for qRT-fPCR (Exon 2)*
AVT_428R	CCAAGGCAGTCAGAGTCCAC	*Reverse Primer for qRT-fPCR (Exon 2-3 Boundary)*

Table 5. *Primers used to clone the AVT mRNA after succesfully isolating two segments of the mRNA using the RACE technique. These primers where designed to flank the Open-Reading Frame of the AVT gene.*

Molecular Genetics

Oligonucleotide synthesis and nucleotide sequencing and analyzes

All oligonucleotide primers used in this study were synthesized by the BioAnalytical Services Laboratory at the Center of Marine Biotechnology. The

particular primers used for PCR amplification and specific cDNA isolation and analyzes (5'-RACE, 3'-RACE and RTFQ-PCR assays) are shown in **Table 5**.

The nucleotide sequences of the cloned cDNA inserts and 5'-flanking region were determined by dye-terminator automatic sequencing (ABI 373 DNA Sequencer STRETCH; ABI, CA, USA). Potential identities of the peptide encoded by each cDNA we isolated were determined by homology search with the Position Specific Iterated-Basic Local Alignment Search Tool (PSI-BLAST) method (Altschul et al., 1997). Nucleotide and peptide sequences were aligned by the CLUSTAL W method (Thompson et al., 1994). All of the above analyzes were performed with the Internet server of the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/).

cDNA Synthesis

cDNA was synthesized using 1 μg of mRNA from each homogenized brain, oligo (dT) primers and the SuperScript II reverse transcriptase (Invitrogen, CA, USA). The reaction was setup according to the manufacturer's instructions. An aliquot of 100 ng of the first-strand from each cDNA was amplified with gene specific degenerate primers. PCR was performed in 25-μl of reaction mixture containing 40 mM Tricine-KOH (pH 8.7), 15 mM KOAc, 3.5 mM Mg(OAc)2, 3.75 μg/ml BSA, 0.005% Tween-20, 0.005% Nonidet-P40, 200 μM deoxynucleotide triphosphate, 2 μM of each primer and 1X Advantage-2 polymerase mix (BD, Biosciences, CA, USA). The PCR conditions were 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min for first 5 cycles; during the remaining 30 cycles the annealing temperature was decreased from 58°C to 52°C. Fragments were purified from agarose gel with QIAquick Gel Extraction Kit (Qiagen Inc., CA, USA), cloned to pGEM-T Easy vector (Promega, WI, USA) and sequenced.

For RACE cDNA preparation, 2 μg of tissue specific poly (A+) RNA was used for constructing 5'- and 3'-RACE cDNA using the SMART™ RACE cDNA amplification kit (BD, Biosciences, CA, USA) following the manufacturer's instructions. AVT-specific primers for 5'-RACE and 3'-RACE, and universal primer mix (UPM) primer (adaptor primer from kit) were used for RACE PCR amplifications with the same PCR reaction mixture described above. Touchdown PCR was utilized at 5 cycles of 94°C for 20 sec and 72°C for 3 min followed by 5 cycles of 94°C for 20 sec, 70°C for 20 sec, 72°C for 3 min, 25 cycles of 94°C for 20 sec, 68°C for 20 sec and 72°C for 3 min. PCR products were separated on agarose gel, purified by QIAquick Gel Extraction Kit (Qiagen Inc., CA, USA), cloned into pGEM-T vector (Promega, WI, USA) and sequenced.

Synthesis of sense RNA standards for RTFQ- PCR

For gene specific RNA standard synthesis, plasmids containing the relevant partial cDNAs encoding open reading frame were linearized and used as the templates. The transcription mixture (20 μl) included 1 μg of linearized template cDNA, 1 mM of ATP, GTP, CTP and UTP, 10 mM of DTT, and 20 units of RNase inhibitor and T3 or T7 RNA polymerase (Invitrogen, CA, USA). Transcription was performed for 2 hours at 37°C. Plasmid DNA template was removed by 1 unit of RNase-free DNase (Promega, WI, USA). The reaction was stopped by adding 40 μl of 5 mM EDTA (pH 8.0), and then purified through a size exclusion column, Chroma Spin-400 (BD Biosciences, CA, USA). The amount of each RNA standard was measured using RiboGreen RNA quantitation kit (Molecular Probe, OR, USA).

Quantification of gene expression at transcript levels

The expression of each gene we investigated in homogenized brain tissue was determined at the transcript level using RTFQ-PCR assays. The basis for this highly sensitive and specific assay is the quantitative increase in fluorescence associated with the binding of SYBR® Green dye to accumulated double-stranded amplification products during PCR (Molecular Probes, OR, USA). Total RNA was isolated from homogenized brain tissue using a modified acid-phenol extraction method, Tri-reagent, (MRC, Inc., OH, USA). The RNA was reverse-transcribed into cDNA using random hexamers and MMLV reverse transcriptase (Promega, WI, USA). PCR was performed in 96-well plates on diluted cDNA templates using SYBR® Green PCR Core Reagent (ABI, CA, USA) and gene-specific primers (**Table 5**). Amplification reactions are carried out via ABI Prism® 7700 Sequence Detection System (ABI, CA, USA), which consists of a built-in thermalcycler, laser irradiation and CCD detector system, and software analysis package. For each reaction, the Prism® 7700 generates an amplification plot of fluorescence signal versus cycle number, and determines C_T, the fractional cycle number at which fluorescence passes a baseline threshold value (Fink et al., 1998). Copy number in unknown samples is determined by comparing C_T values to specific RNA standards and values are normalized to the amount of 18s RNA in each sample. In some instances, an additional normalization step was performed in accordance to ABI recommendations. In this added normalization step, the copy number of the gene studied is normalized using the copy number of the 18s RNA that had the highest expression in the group. The data is divided into groups consisting of the different sampling months.

Immunocytochemistry

Brain sections cut at 6 μm were deparaffinated and rehydrated. Each section was incubated with 0.5% H_2O_2 for 30 min, washed for 5 min in PBS, treated with 0.5% Triton X-100 for 10 min, washed for 5 min twice in PBS and blocked for 30 min at 25^{o}C with blocking buffer (3% goat serum and 2% blocking reagent in PBS). Sections were then incubated for 48 H at 4^{o}C with a 1:4,000 dilution of a commercial rabbit anti-mouse AVP antibody (Invitrogen). The peptide identity of mouse AVP to seabream AVT is 89% (Elizur et al., 1996) and the cross reactivity of anti-rat AVP to fish AVT has been demonstrated (Foran and Bass, 1998). Excess antibody was removed by two 15 min washes in PBS. Using Vectastain *Elite* ABC-Peroxidase Kits (Vector, CA, USA), biotinylated goat anti-rabbit IgG secondary antibody, diluted in blocking buffer 1:200, was added to each section. After a 30-min incubation, excess antibody was removed by two 15 min washes in PBS and the ABC method was applied according to the manufacturer's instructions. Color development in sections was initiated with 3, 3-diaminobenzidine tetrahydrochloride (DAB; Sigma, MO, USA) at 25^{o}C. After DAB color development, sections were rinsed for 5 min in water, counterstained with either 0.02% methyl green or 0.02% fast green in 0.02% acetic acid for 5 min and dehydrated through successive baths of ethanol (50, 70 and 95 %). Sections were mounted in aqueous mounting medium (Mount-quick, RPI, IL, USA), examined under a light microscope and computer images were taken as described above.

Quantification of Serum Estradiol and Testosterone

Serum levels of estradiol and testosterone will be measured using a RIA kit, which is commercially available (DPC, Los Angeles, CA). These RIA kits have been

previously validated for measurements of plasma testosterone, estradiol, and cortisol in the gilthead seabream (Gothilf et al., 1997).

Real Time Quantitative Fluorescent PCR

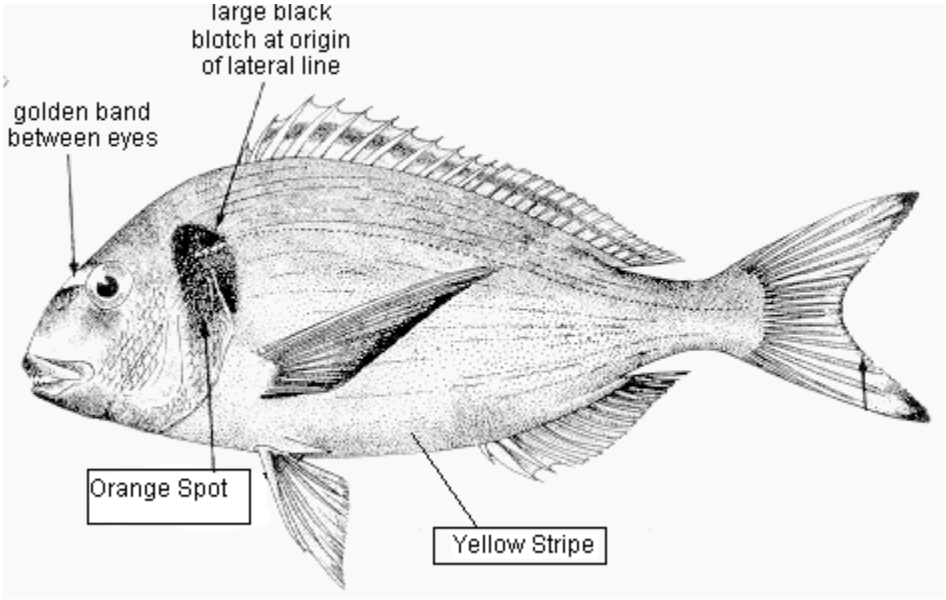


Figure 28. Color markings in the Gilthead seabream, *Sparus aurata*. Both the "Orange Spot" and the "Yellow Stripe", seem to change intensity in preparation to, and during, spawning. Modified from a picture in *http://www.fishbase.org*

Using the sequence information from the cloned AVT mRNA, and information from the known mammalian AVP sequences, I identified the exon-intron boundaries in the transcript. I designed and tested several primer pairs that did not generate any genomic DNA amplification. After the best primer pair was identified, I established an RT-QFPCR protocol for measuring the levels of AVT mRNA in homogenized seabream whole brain tissue.

Measuring of Pre-spawning and Spawning Coloration

Gilthead seabream have two visible color spots that appear around the onset of spawning in young fish (**Figure 28**). Older fish may have these markings year-round, but

they seem to be more intense around spawning (Pers. Obs.). The first, and most prominent, is an orange spot that appears just anterior to the operculum of the fish. The second marking is a yellow stripe that appears only around the time of spawning. It runs along the side of the "belly", between the pelvic and the anal fins, around the region where the gonads are located. The stripe is about 3cm

to 6cm in length, depending on the size of the individual. All Gilthead seabream also have a yellow stripe between the eyes, which gave the fish its latin name, *Sparus aurata*.

Color intensity was measured subjectively, using a scale from 0 (absence of markings) to 3 (full intensity) by an experienced researcher who had worked with seabream for some time before performing these observations (T.T. Wong).

Results

AVT

Cloning of the Seabream AVT mRNA

The complete open-reading frame was identified using primers AVT21F and AVT521R, which were designed, based on AVT sequences from other related fish species. Once the AVT mRNA was cloned, it was possible to identify the 3 exons present in AVT mRNA vertebrate sequences (**Figure 29**). It is important to identify the exon-exon boundaries, because placing the amplicon primers within these boundaries precludes amplification of genomic DNA. Thus, using this information, primers for the Q-RT-PCR were designed. The clone itself was used to create the standards for the Q-RT-PCR.

According to a phylogram that I constructed using all the NCBI-published sequences for fish AVT and Isotocin (another oxytocin-like peptide found in fish), the

seabream AVT mRNA is most closely related to the sequence found in another sex changing fish, the bluehead wrasses *Thalassoma bifasciatum* (**Figure 4**). In both of these fish, AVT is more important for the control of reproduction and sex-specific behavior.

5'-CCGCGGGAATTCGATTCCTCGCAGCAGGACATACAGGTGCGGTCGCGCTCATCCACA
ACCAGCCAGCAGCGATGCCTCACTCCTTGTTCCCCCTGTGCGTCcTGGGACTCCTTGCG
TTCTCCTCTGCCTGCTACATCCAGAACTGCCCCCGAGGAGGGAAGCGGGCGCTGCCAG
AGGCTGGGATCAGACAGTGCATGTCGTGTGGCCCCAGAGACAGGGGCCACTGTTTCGG
CCCCAACATCTGCTGCGGGGAGGGCCTCGGCTGTCTGCTGGGCTCCCCGGAAACAGCT
CACTGTGTGGAGGAGAACTACCTGCTCACCCCCTGCCAGGCGGGAGGGAGACC
CTGTGGCTCTGAAGGAGGACGCTGCGCTGCTTCAGGACTCTGCTGTAACT
CAGAGAGCTGTACGGTGGACTCTGACTGCCTTGGGGAGGTTGAGGCCT
CAGACCCGTCCGACAGCTCTGCGGGGAGCTCGCCTGCAGAGCTGCTGCTGCGCCTGCT
ACATGTGGCCACCAGAGGACAGACCGAGTACTGACGCTGTCGCCTGCGGAGCCTCTTC
TgCCTCTCAGGCCCtGGAGGTGCAGAATGAACATCATCCCTGTTCCACTATAAGCCTTG
AGATTTGAACCCTGAACCAATAAAATGCCCAATCACTAGTGAATTC-3

Figure 29. Seabream AVT mRNA sequence. Exons A, B and C are shown in yellow, green and blue respectively. The primer sites used for Real-Time PCR are shown in a larger font size. Notice that at least one primer lies in an exon-exon boundary (Total PCR Amplicon size is 81 bp).

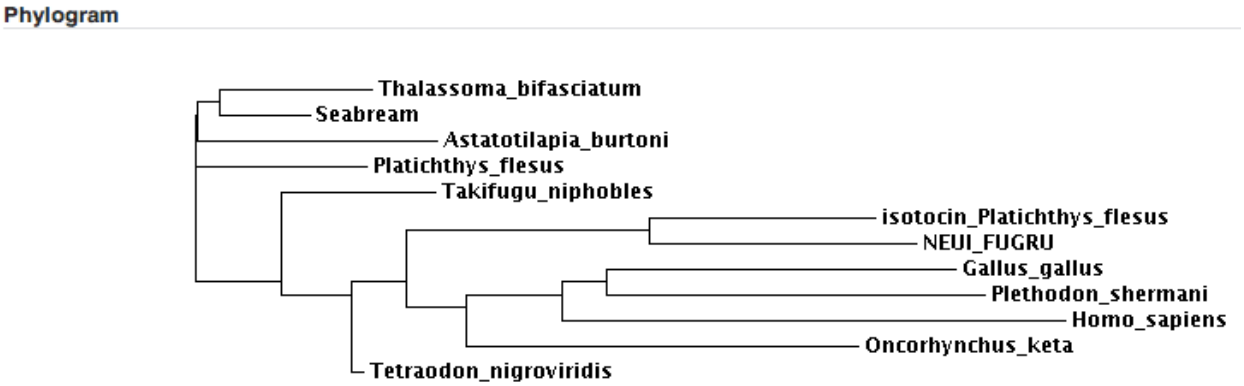


Figure 30. Phylogram showing the relationship between Gilthead seabream, *Sparus aurata*, AVT mRNA and other mRNA in the PubMed database, including various representatives of Tetraodontiformes and Perciformes. The phylogram also includes the more derived AVP from *Gallus gallus* and *Homo sapiens* and isotocin, a related peptide from *Platichthys flesus*.

Immunolocalization Studies

Once the presence of the transcript was confirmed through RACE PCR screening, it was important to show that the protein product was expressed in sexually dimorphic brain structures, such as the Preoptic Area (POA) of the hypothalamus. In clownfish, wrasses and gobies, this region has been shown to be sexually dimorphic for both AVT and GnRH. Anti-AVP has been shown to cross-react with, and label, AVT cells in fish brains (Goodson and Bass, 2000). Thus, I choose to use a commercially available rabbit anti-AVP immunocytochemistry assay, which I modified and validated for use in seabream brain tissue. AVT-immunoreactive (AVT-ir) cells were observed in different regions of the POA. In females the more dorsal region of the POA showed many of these cells and their axons could also be seen (**Figure 31A**). The cells were most likely gigantocellular POA cells (gPOA). The axons of these cells followed a preoptico-hypopheseal tract. In males, the stained AVT-ir cells were present in a similar region, with no axonal staining (**Figure 31B**). A prominent group of cells, located along a tract inside the hindbrain was only observed in the brain of one running male (**Figure 31C**). Above this tract, a large group of axons was observed. Their location and extensive projections suggest that these cells could be involved in motor pathways.

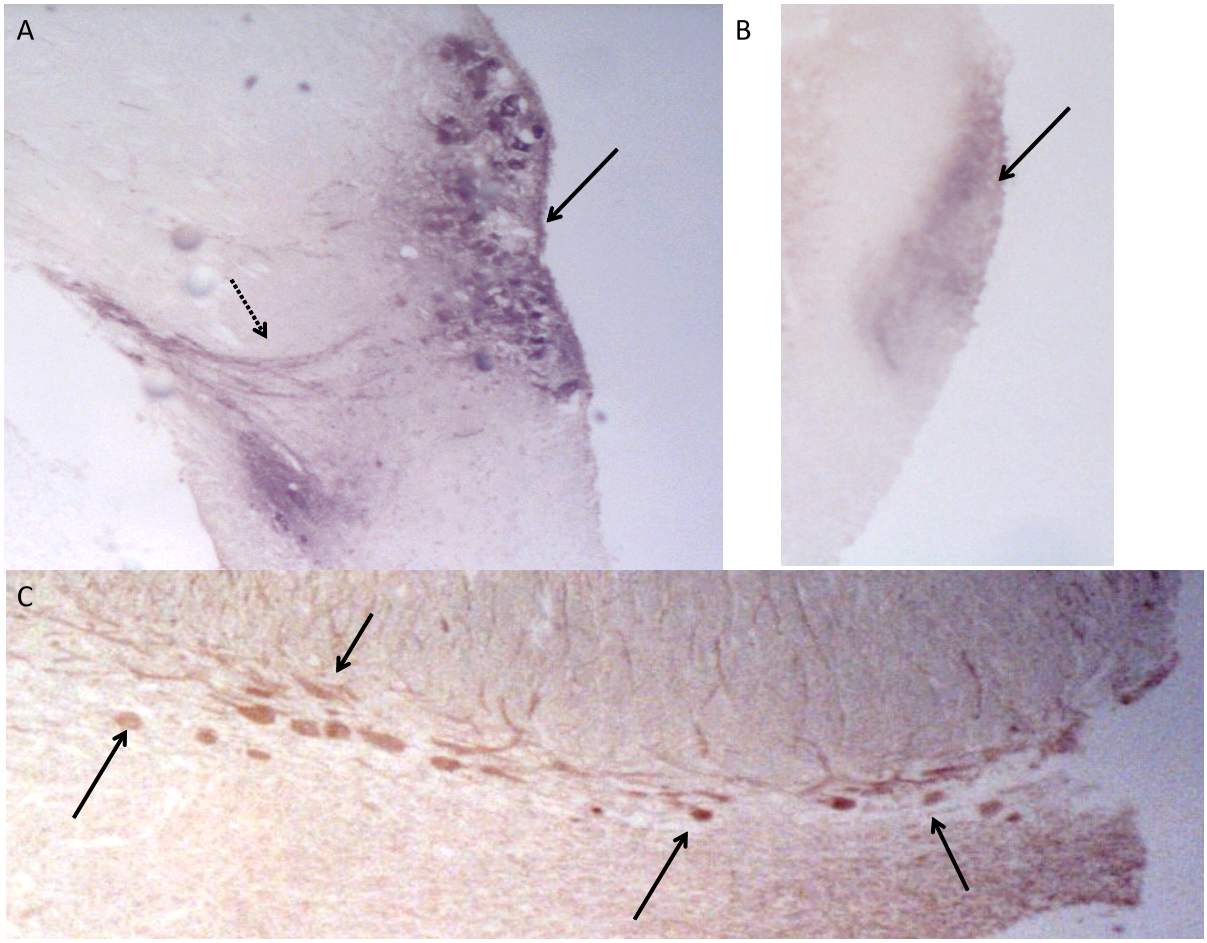


Figure 31. Saggital sections of the brain of the Giltehead seabream showing AVT-immunoreactive somata and fibers. (A) Spawning female. The solid arrow points to the cell bodies in the POA, while the dotted arrow points to the axons of these neurons which probably travel to the pituitary. (B) Spawning male. Hypothalamic region. Arrow points to the AVT-ir cells. (C) At least one running male had a prominent group of AVT-ir neurons located in the hindbrain. Above the tract, some axons can be seen, which suggest that these might be cells involved in motor pathways.

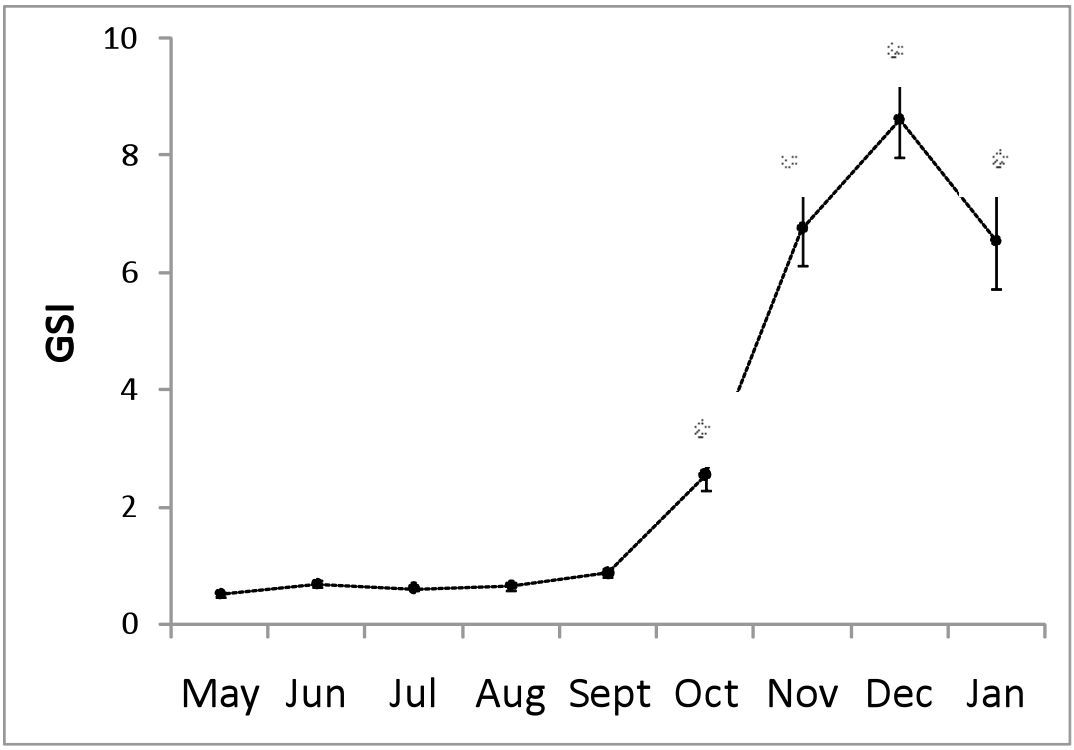


Figure 32. Gonadosomatic index as a function of month of year. Notice that peak GSI occurs around the month of December. The first significant increase in GSI occurs in September. Asterisk mark points significantly above May (Kruskal-Wallis; $p<0.05$)

Gonadosomatic Index

Gonad development begins in earnest around the month of August, when the GSI begins to increase, although not statistically significant. GSI begins to rise significantly in October, and the general trend was for GSI to continue increasing until the spawning season begins (**Figure 32**). GSI closely followed the seasonal profile of serum steroids,

and was correlated to serum estradiol levels (**Figure 33** and **34**).

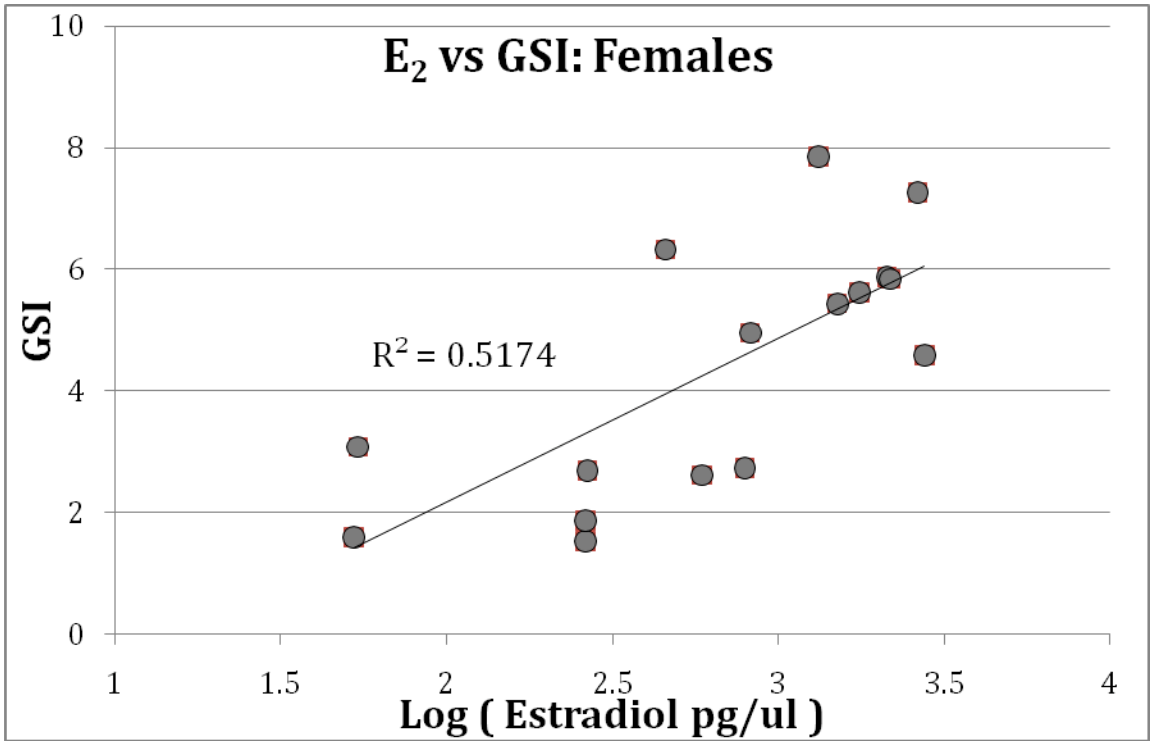


Figure 33. GSI correlates very closely with estradiol expression in females. As GSI increases, and the gonad is larger, the amounts of steroids produced by the gonad also increases. Indeed, estradiol is a good indicator of gonad development in *Sparus aurata*.

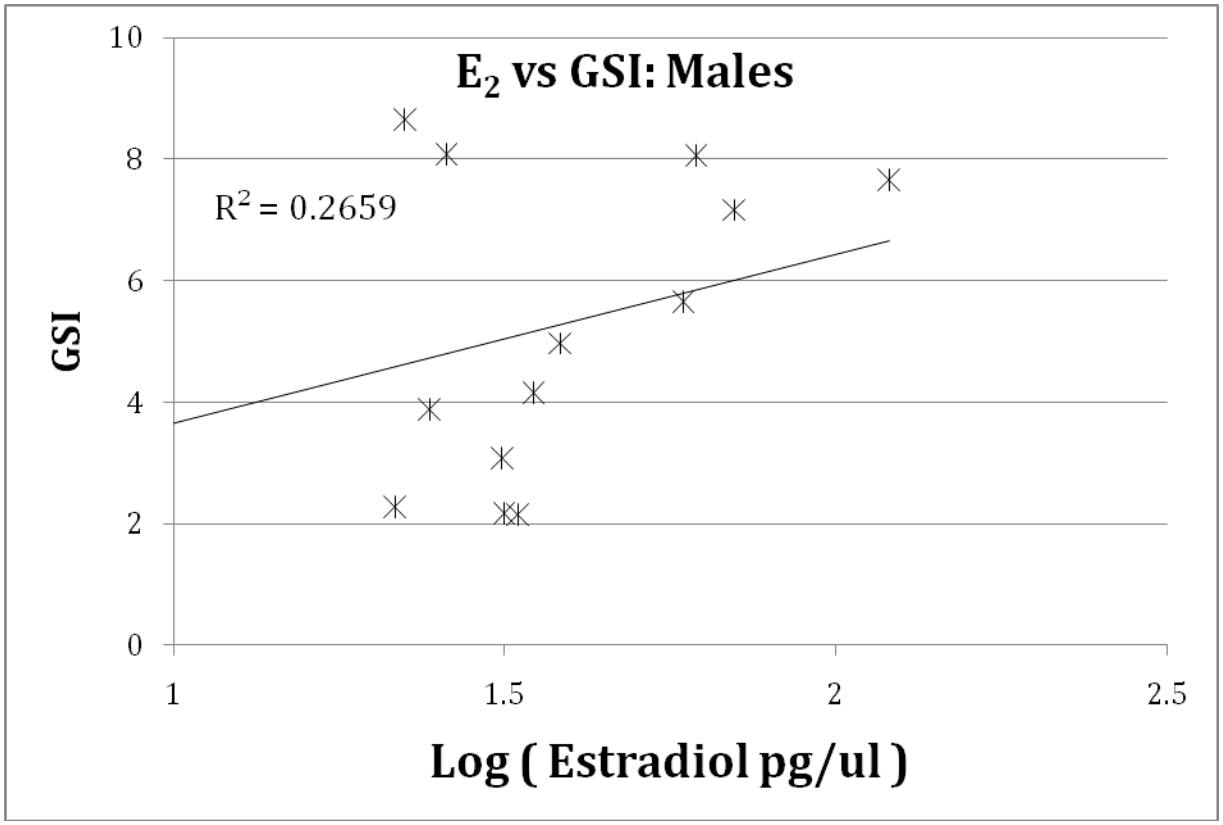


Figure 34. GSI correlates very closely with estradiol expression in males. As GSI increases, and the gonad is larger, the amounts of steroids produced by the gonad also increases. Indeed, estradiol is a good indicator of gonad development in *Sparus aurata*.

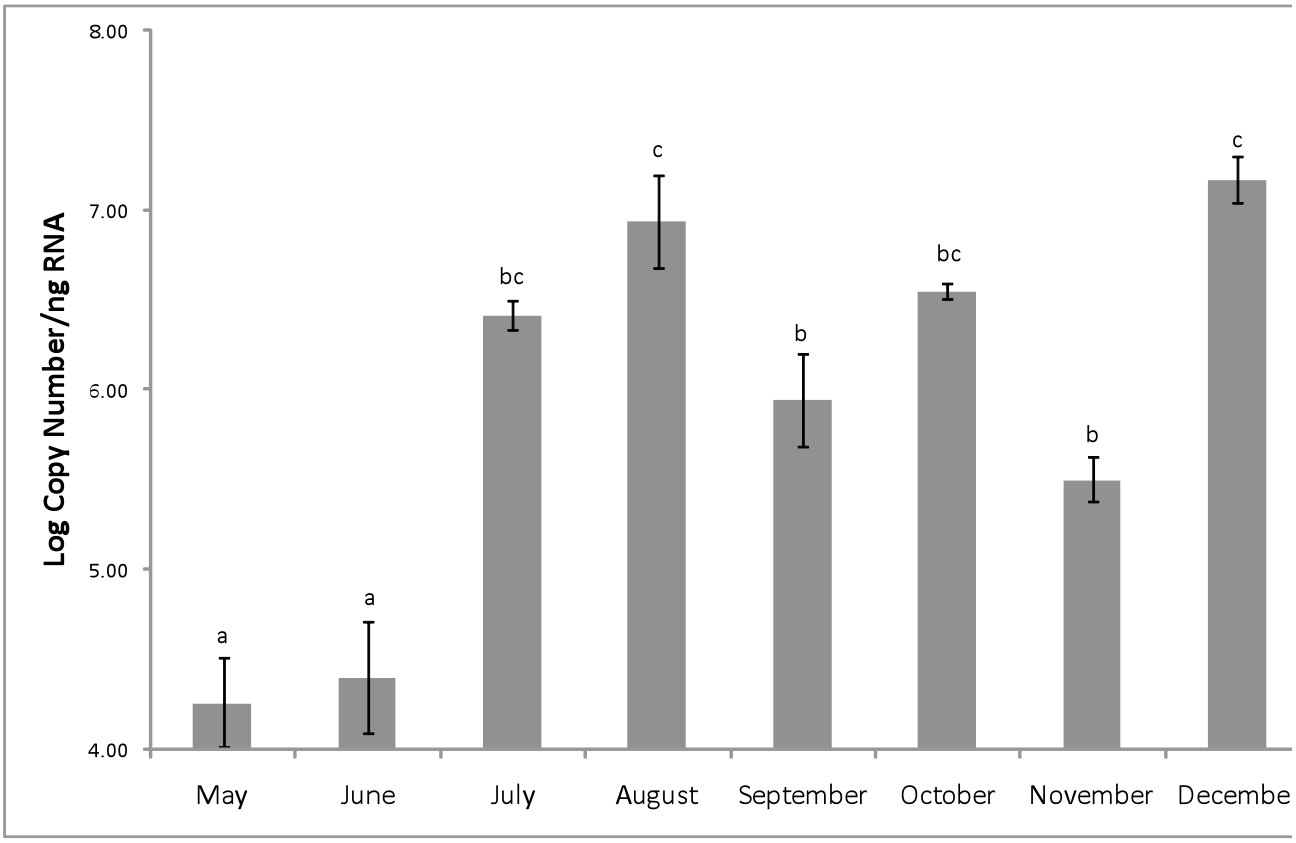


Figure 35. AVT expression in whole brains during the seabream gonad cycle. Note that during the summer quiescence, AVT expression is at its nadir, but rises quickly during July and August. AVT expression also rises in December, at the onset of the reproductive season of this species.

Seasonal Expression of AVT mRNA in the Gilthead Seabream During the Gonad Cycle.

AVT mRNA expression changes significantly during the gonad cycle (**Figure 35**; F=28.345; N=83; $p<0.0001$). Applying a Tukey-Kramer post-hoc test to the data revealed that its expression level is at its nadir during the months of May and June. In July AVT starts to increase significantly and peaks in August. AVT mRNA expression in the whole brain drops significantly from August to September, and it does not increase again until December. Just before this second peak, the levels of AVT seem to drop to a second nadir, during November.

GnRHs

The three known isoforms of GnRH in seabream brain were cloned previously by members of the Zohar group (Gothilf et al., 1997; Elizur et al., 1996). Based on these sequences, Wong et al. (2004) developed a specific RT-QF-PCR assay for these genes.

GnRH-1 expression in the brain of Gilthead seabream remained unchanged until December, when mRNA levels in the brain peaked significantly above the levels observed in the previous five months(**Figure 36**; F=73.544; N=83; $p<0.0001$). GnRH-2 exhibited a similar pattern, with unchanged expression levels for the first five months, but an increase after December (F=29.524; N=71; $p<0.0001$). GnRH-3 levels peaked twice: the levels of GnRH-3 increased significantly from July to August, and from November to December, while remaining unchanged during the months of July, September, October and November (F=16.760; N=69; $p<0.0001$).

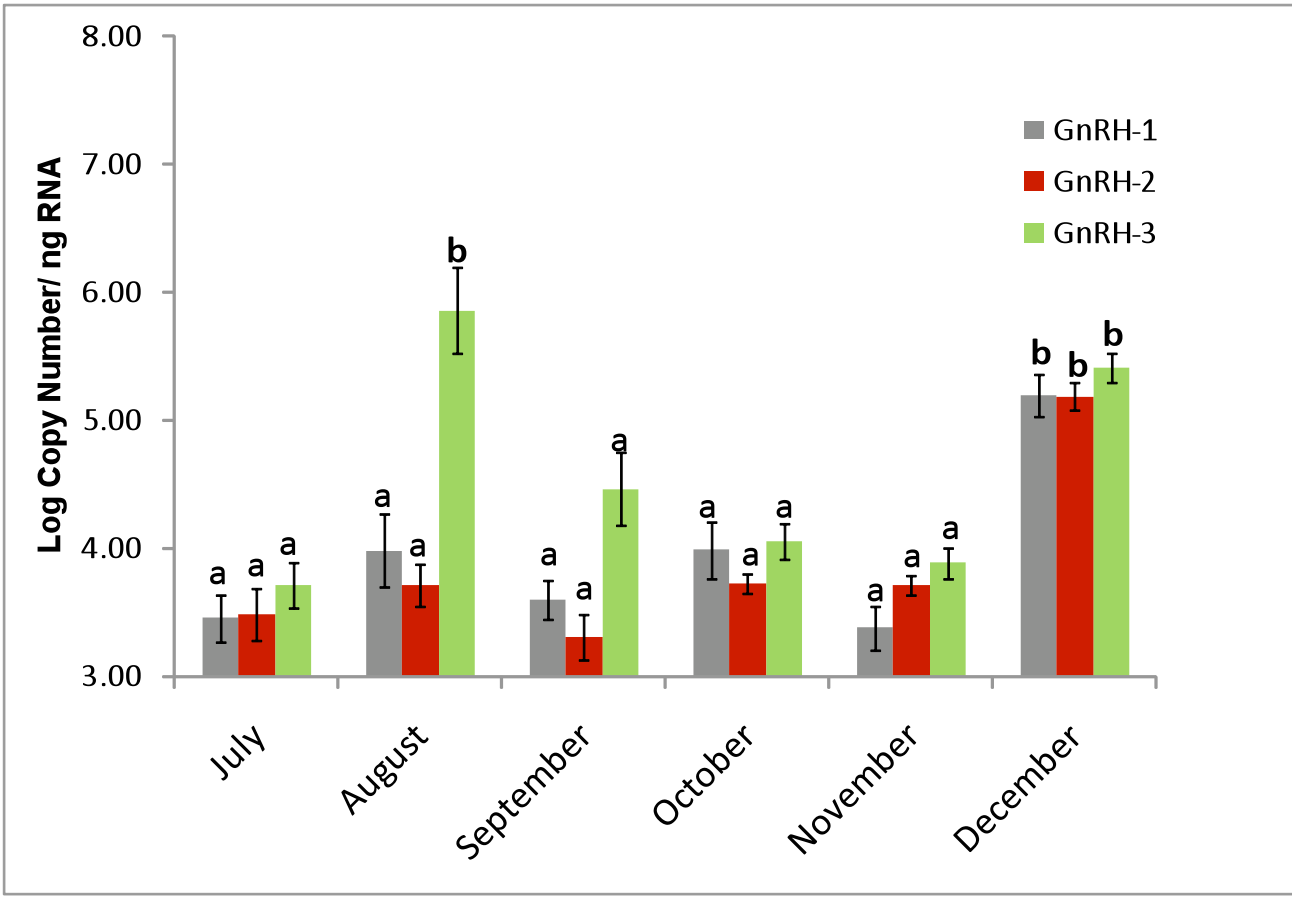


Figure 36. Expression levels of the 3 GnRH isoforms present in whole brain extracts of Gilthead Seabream throughout the gonad cycle. Notice that GnRH-3 levels increase significantly in September, then decrease. GnRH-3 increases again in the month of December, when all of the GnRHs also increase.

Correlated Expression of GnRH-1, GnRH-2, and GnRH-3 with AVT.

In the brain, the levels of GnRH-1 mRNA did not show any significant correlation with the expression levels of AVT mRNA (**Figure 37A**; Spearman r=-0.07626; N=97; p=0.458). Similarly, the expression of GnRH-2 mRNA in the brain was not correlated to AVT expression (**Figure 37B**; Spearman r=0.1764; N=99; p=0.081). However, GnRH-3 expression showed a positive and significat correlation with the expression of AVT mRNA (**Figure 37C**; Spearman r=0.4484; N=98; p<0.0001).

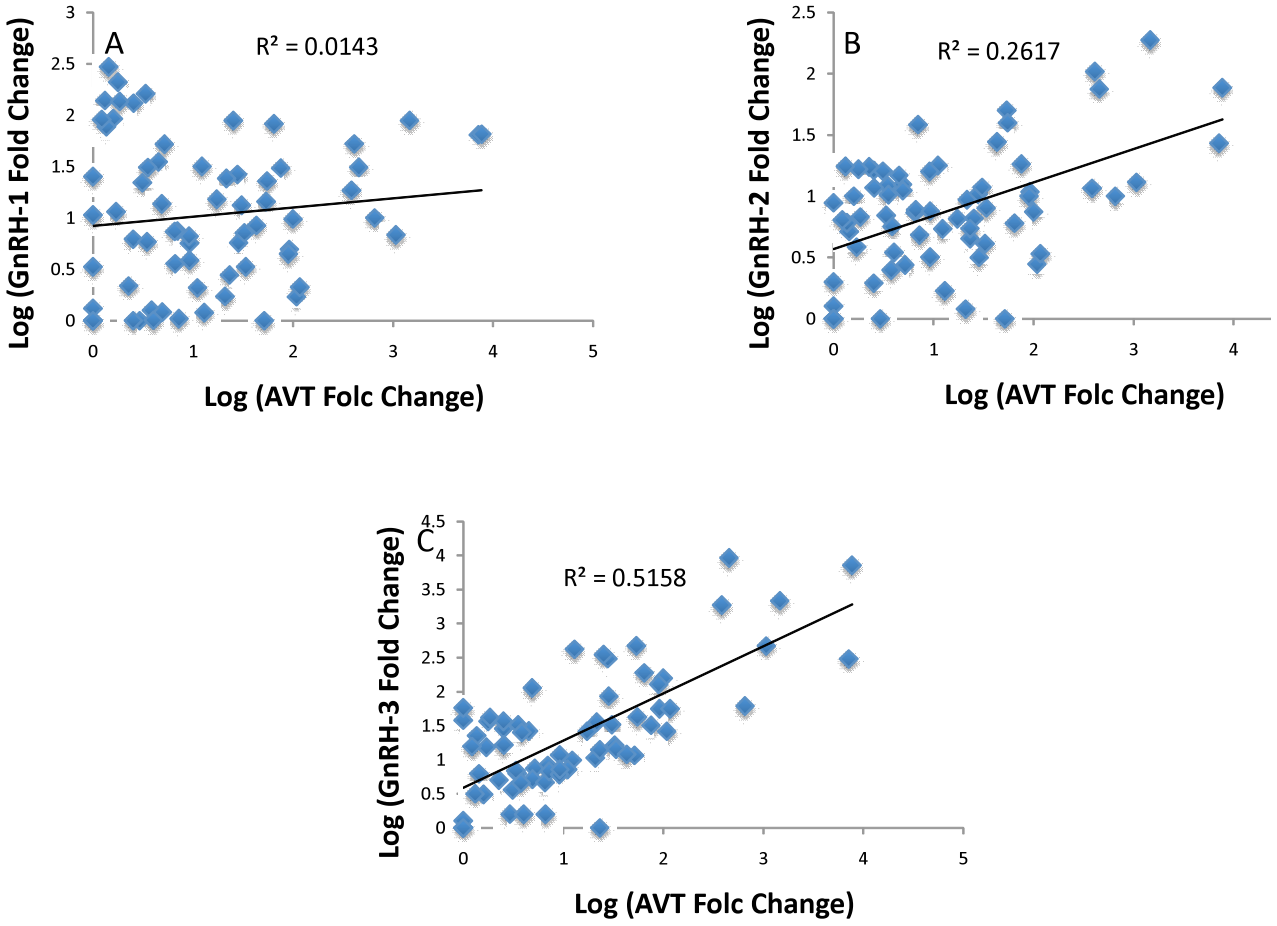


Figure 37. Expression levels of AVT mRNA versus the mRNA for the 3 endogenous forms of GnRH found in Gilthead seabream. Only GnRH 3 vs. AVT showed a significant correlation ($p<<0.001$).

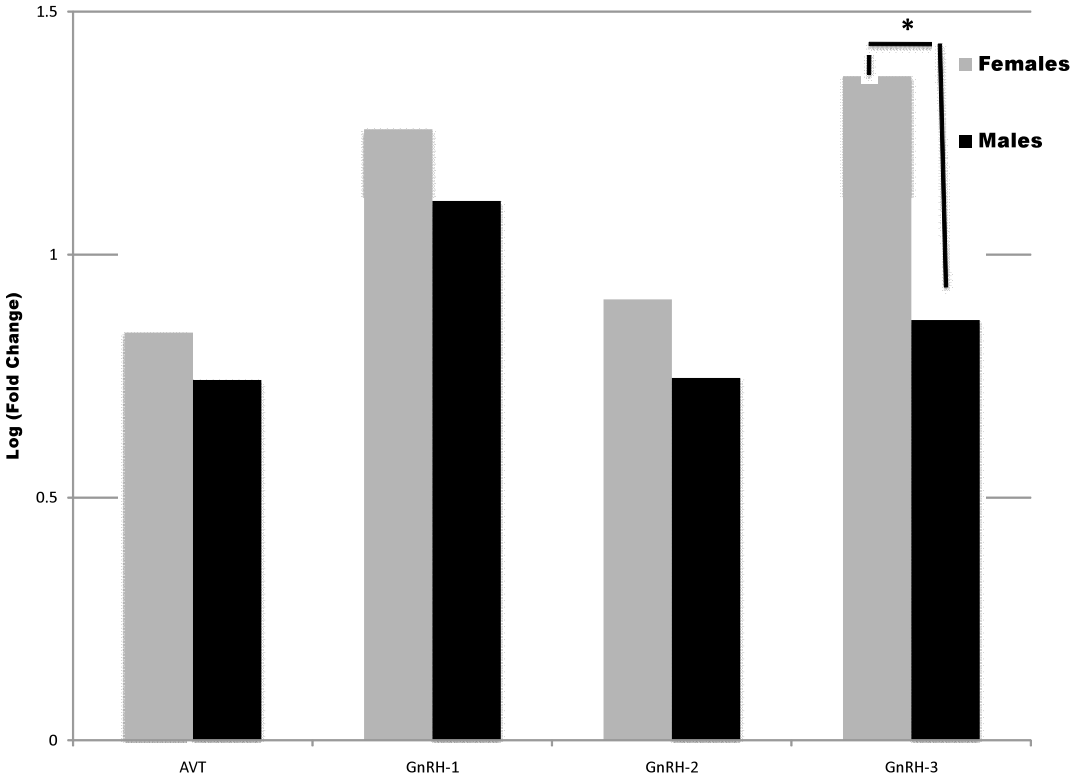


Figure 38. Sex differences in the pattern of mRNA expression among male (N=24) and female (N=24) seabream. The only significant difference was in the content of GnRH-3 among males and females ($p<0.01$).

Sex Difference Among AVT, GnRH-1, GnRH-2, and GnRH-3.

To test if any of the neuroendocrine factors of interest was differentially expressed between females and males, the expression levels for October, November and December were averaged. Since each sampling period had 8 animals of each sex, the total number of individuals for this test was 24. Only GnRH-3 showed a significant differential expression between sexes (**Figure 38**). GnRH-3 expression in the brain was significantly higher among females, when compared to males ($t=2.826$; $p<0.01$). All of the other peptides measured showed a consistent trend of increased expression in females

when compared to males, although these differences were not significant at the 0.05 level.

Seasonal Levels of Estradiol and Testosterone Throughtout the Gonad Cycle

As early as June, some ambisexual individuals had low but detectable levels of serum estradiol. Afterwards, as gonad development progressed, estradiol levels were significantly higher in females (**Figure 39**). While both spawning males and regressed females had some detectable serum estradiol (E_2), the hormonal profile during spawning (i.e. December and January) was unique for each sex: males had detectable, albeit very low, levels of testosterone, and most had low or undetectable levels of E_2; Females on the other hand, had very high levels of E_2 but very low or undetectable levels of testosterone (**Figure 39**).

During the summer months (May-July), the levels of testosterone and E_2 reach their nadir (Testosterone is below detection and $E_2$is around detection limit). As the gonad develops during the month of August, the levels of E_2 start increasing (July = 6.59 pg/ul; August=28.57pg/ul). This may be due to the fact that in seabream, the ovarian portion of the gonad begins developing before the testicular portion. In September, the levels of testosterone begin to rise (August=0; September=2.16). After October, and throughout the spawning period, the levels of testosterone and E_2in the serum increase and reach their peak around the month of December. This coincides with the peak in Gonadosomatic Index.

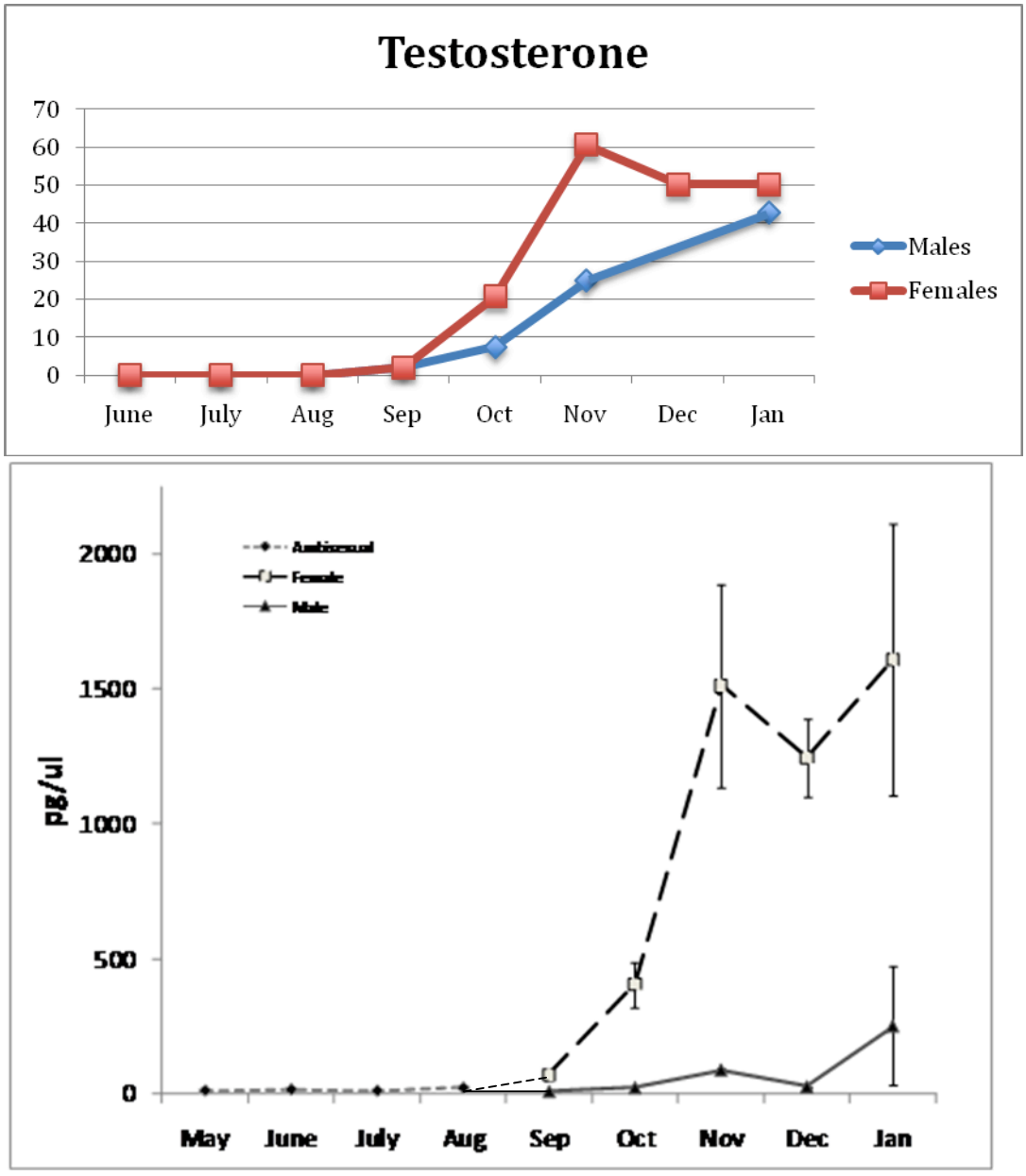


Figure 39. Total serum steroids as measured by Radio Immunoassay. Testosterone (A) and E_2 (B) peak in females around the month of November and remain elevated throughout spawning. In males, serum estradiol remains low throughout the year. The hormonal profile is clearly different between females and males. Peak concentration of steroids do not coincide with spawning but with the onset of gonad development, suggesting that they play an important organizational role.

Pre-Spawning and Spawning Coloration

Beginning in the month of July, some individuals began showing an increased in the pigmentation intensity of their yellow belly strip and orange opercular spot (**Figure 40**). The intensity of this spot seemed to increase every month until October, when it reached its peak. Afterwards, intensity of this marking decreased. The yellow marking behind the pelvic fin seemed to follow a similar trend, with a peak in intensity around October in the males. However, after October, the intensity of these marks seemed to drop in the males, but in females it continued unchanged through spawning.

The orange opercular color was significantly more intense in females than in males, regardless of sampling month (Kruskal-Wallis Test; N=67; Rank Difference=33.07; $p<.001$). The same was true for the yellow strip across the abdomen (Kruskal-Wallis Test; N=67; Rank Difference=46.26; $p<.001$). Thus, females were the most intensely colored of the two sexes. Similarly, ambisexual individuals at earlier stages of development (i.e. fish sampled before sex was evident), were significantly less colorful than females (Kruskal-Wallis Test. N=76. Yellow Marking: Rank Difference =-34.56; $p<0.001$. Orange Marking: Rank Difference=-16.99; $p<0.001$). The difference between the color intensity of males and ambisexual fish was not significant (Kruskal-Wallis Test. N=76. Yellow Marking: Rank Difference = 11.701; $p>0.05$. Orange Marking: Rank Difference=16.087; $p>0.05$). However, there was a trend of higher intensity among the ambisexual fish, for both the yellow and orange markings, when compared to males.

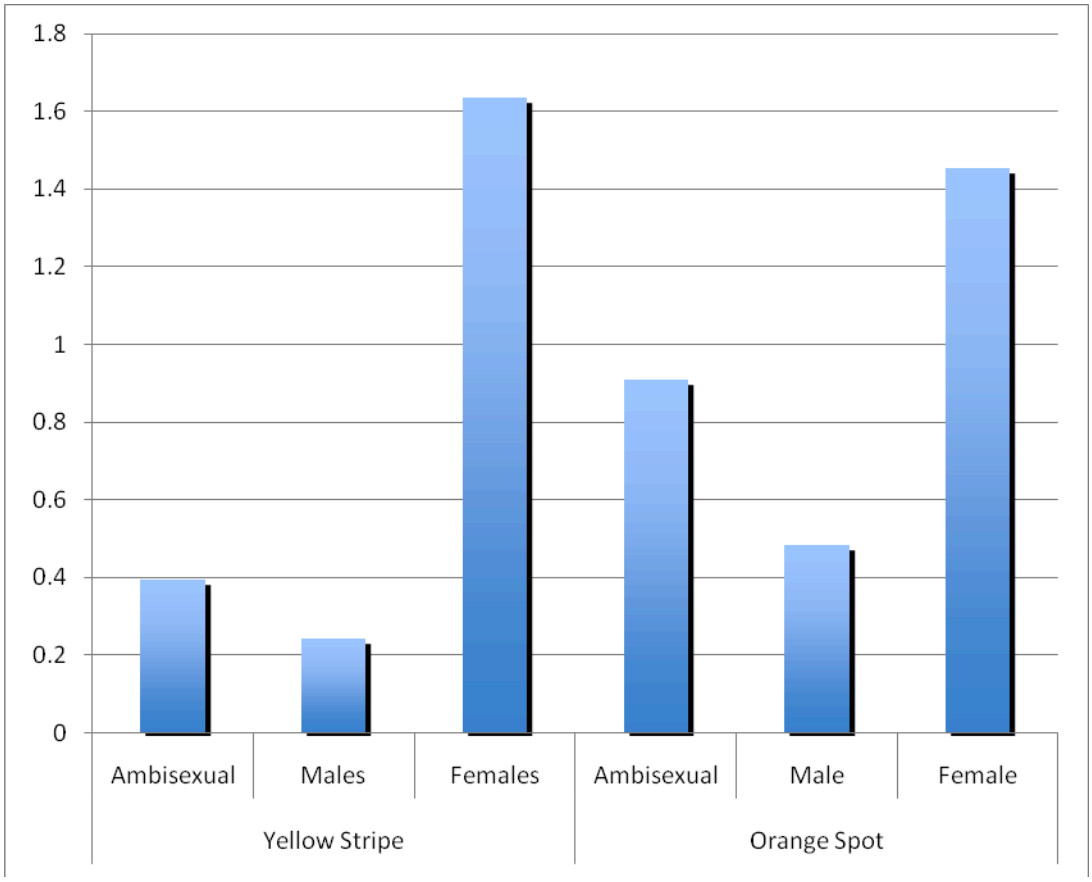


Figure 40. Average color intensity of the two markings observed in seabream. Note that they are sexually dimorphic. The female is the most colorful of the two sexes. A score of 0 = No marking; 3 = Full intensity.

Discussion

This study is the first to describe the seasonal expression profile of the three GnRHs and AVT in the brain of the Gilthead seabream. For GnRH, the expression profile seems to be similar, but not identical, to that of the black porgy, a protandrous hermaphrodite that also spawns in the winter and early spring (Du et al., 2005). Like juvenile black porgy, ambisexual seabream show a peak in the expression of brain GnRH-3, just at the onset of gonadal sex change which occurs around the month of August. GnRH-1 and GnRH-2 expression levels did not change significantly during the gonad sex change period. All three GnRHs increased prior to spawning in December.

This is similar to what Ghotilf et al. (1997) showed in pre-ovulatory female seabream, in which all the GnRHs also increased just before ovulation,

GnRH-3 is present in the olfactory bulb of seabream (Ghotilf et al., 1996), where it is thought to act as a modulator of behavior, by participating in the detection of olfactory cues that might coordinate reproduction (Zhang and Delay, 2007). Because it seemed to increase significantly at the same time or just prior to gonadal development, it is possible that this GnRH might be involved in this process. Perhaps, it coordinates pheromonal or visual cues that control sex ratios. For example, Happe and Zohar (1988) have suggested that sex change in the Gilthead seabream is regulated by water-borne pheromones. Although their highest sensibility is to gastrointestinal fluids, seabream are sensitive to olfactory cues (Hubbard et al., 2003). Removal of males in from a spawning group, leads to significant changes in the HPG axis of females, including the GnRH system (Meiri et al., 2002). The sensory modality involved in this previous study is unknown, although the authors hypothesized that visual or pheromonal cues were involved. Similarly, Fernald (2003) has suggested that in Tilapia, pheromones may also play a central role in social modulation of gonadal development. Experiments by Wong (unpublished) in which he showed that sex change can occur in sensory isolation, but with inconsistent results, seem to be at odds with this hypothesis. However, Wong's attempts to isolate water-borne pheromones might not have been effective, as he used foam fractionation and charcoal. Alternatively, the isolation of the animal might be too stressful and sex change (or its suppression) under such conditions might be extra-physiological. Nevertheless, the fact is that GnRH-3 might be an important mediator of sex change. What environmental cue it translates or is sensitive to, is still unknown. It

has been suggested that GnRH-3 transduces other sensory modalities, including odor and visual cues.

The importance of GnRH-3 in this process was further emphasized by the fact that only GnRH-3 showed significant differential expression between sexes. Indeed, it is consistently sexually dimorphic, even when the whole brain was assayed. Because it showed higher levels of expression in females than in males, and because females seemed to show pre-spawning coloration to advertise their sex change, it is possible that GnRH-3 plays a role in translating such visual cues. I expected GnRH-2 and AVT to be expressed differentially among males and females. This hypothesis was not supported by the statistical analysis of the results, although it seemed that all the genes tested showed a trend of higher expression among females. Thus, instead of focusing on GnRH-2 or AVT as possible mediators of the behavioral changes observed in seabream after sex change, it seems that GnRH-3 should be emphasized. Perhaps the results only show that GnRH-3 translates a pheromonal cue that affects sex change. However, even if GnRH-3 is important to effect behavioral sex change in the brain, the fact that all 3 GnRH's showed a trend in increase expression in the females suggest that GnRH-3 is not the only GnRH that could be differentially expressed among males and females after sex change. This fact is especially true when one considers that these assays where performed using the whole brain, as opposed to cutting the brain and attempting to quantify more localized expression (e.g. forebrain or hindbrain expression). By definition, such alternate techniques might be more sensitive to differences in expression levels at specific regions in the brain.

As expected, this study confirmed the presence of AVT in the brain of the hermaphroditic Gilthead seabream. AVT seems to be produced mostly by the cells in the POA nuclei of the hypothalamus, which is also the site of production of GnRH-1, the natural inducer of gonadotropins in seabream. AVT is highly conserved in fish, and seabream's deduced AVT peptide sequence is identical to other published fish AVT peptide sequences. Phylogenetic analysis of the AVT open-reading frame sequence showed that it was closer to wrasse's AVT than any of the other bony fishes published sequence.

This study is the first to describe the seasonal expression of AVT in the whole brain of a protandrous hermaphrodite using the highly quantifiable fRT-PCR. During the summer months, when the gonad is regressed, AVT expression is low. In July, AVT expression starts to increase. Interestingly, this coincides with a period of increased dominance behavior in the fish (Chapter 2 and 4). AVT mRNA expression levels in the whole brain peaked significantly in the following month, August. At first, this seemed like an oddity. However, GnRH-3 followed a similar pattern of seasonal expression, with a peak in August. It is possible that this is associated with the development pattern of the gonad, and/or its increased production of gonadal steroids. The seabream gonad begins to differentiate into male and female gonad, during the months of September and October. Thus, the seasonal expression of AVT may be following very closely the gonad development itself. Alternatively, the rise in AVT and GnRH-3 expression could be related to behavioral events that occur during these months and which affect sex change. During the summer, hierarchies in seabream seem to be more linear and stable (Chapter 2 and 4). AVT has been shown to stimulate aggressive and dominance behaviors in many

vertebrates (Review: Rose and Moore, 2002). Aggressive behavior is one way in which social context might influence sex change.

GnRH-3 is associated with sensory pathways that affect the reproductive axis (Review: Zohar et al, 2009; Review: Oka, 2009). Perhaps, GnRH-3 expression levels increase as the animal's need to assess its social environment also increase. Because upregulation of both neuropeptides occur close to the beginning of the sex-changing season, and prior to gonadal commitment, it is likely that they play some role in the process of gonad development or gonad sex change. This experiment was not designed to test which of these two roles it might have or its causal relationship with these processes.

AVT expression patterns were different between males and females. Gigantocellular AVT-ir POA cells (gPOA), were more prominent in females. AVT-ir cells in the hindbrain were almost exclusively observed in males, with at least one spermiating male showing a very clear neuronal tract along the hindbrain with extensive axonal projections. These observations are in agreement with previous work in other teleost species (Maruska, 2009; Maruska et al., 2007; Grober et al., 2002; Godwin et al., 2000; Foran and Bass, 1998). Recently, Maruska (2009) showed that the forebrain is not the only region were sexual dimorphism exist in the expression pattern of AVT. Indeed, the author points to a neuronal tract inside the hindbrain which also has extensive projections, and which, perhaps by coincidence, is also only observed in males.

AVT expression levels dropped to a significant nadir in November, but quickly peaked the following month of December. It seemed that all three GnRHs also increased their expression levels during December. It is possible that a general increase in mRNA occurs during spawning in all of these genes, as they are involved in different aspects of

reproduction. What roles each of them play in the reproductive biology and behavior of seabream is not known, but experiments in other species show that AVT, GnRH-2 and GnRH-3 are associated with reproductive behaviors (Fernald, 2003; Godwin and Semsar, 2003; Pickford and Strecker, 1977) and GnRH-1 is important in the activation of gonadal development and gamete maturation.

According to their individual seasonal profiles, GnRH-3 and AVT seasonal expression seemed to follow each other. A Spearman's rank correlation was used to test this hypothesis. It showed that indeed, GnRH-3 and AVT mRNA expression are significantly correlated. This correlation was not significant for GnRH-1 or GnRH-2. Correlation does not imply a causal relationship, but it suggests that perhaps both neuropeptides are important in organizing behavioral or gonadal sex change. The experiment presented here was not designed to address the relationship between AVT and GnRHs, but future experiments could address this question.

Wong (2003) showed that during the month of July, the expression of gsbCYP19A1 aromatase, the gonadal aromatase that catalyzes the conversion of androgens to estrogens in the Gilthead seabream, increases in the ambisexual gonad. He noted this event as the possible point of committal for the gonad: the putative sensitive period which Zohar (1978), based on gonad histology and his extensive work with seabream, had proposed. Wong then cites work by Lee et al. (2002) who showed that blocking aromatase from April to October in the black porgy successfully precludes sex change. Based on these findings and the seasonal expression of the gonadal aromatase, he surmises that "June and July may be the very sensitive window for the sex-reversal process".

Based on the data that I have shown here, I believe this conclusion needs to be modified. The putative sex change window, if it exists at all, is not limited to the summer months. The data I have analyzed can be used to extend the time frame of sex change in this species and to propose a revised, but provisional, model that takes into consideration these findings (**Figure 41**). The basis for this model is several facts, previously unknown, and which I uncovered here.

The first line of evidence is the rise in brain AVT levels that occur in the month of July. As discussed above, gonadal aromatase, a marker for gonad development, also reaches its peak expression in the month of July then drops back to pre-July levels during the month of August, September, and October (Wong, 2003).

In August, September and October, gonadal aromatase in the ovarian portion of females and males is similar. Starting in the month of November, the gonadal aromatase in the developing ovary is significantly higher in females than in males (Wong, 2003). Around this time, aggressive behavior is usually lower and no true dominance hierarchies are observed among fish in groups (**Chapter 2**). Together, these data suggest that gonad development remains uncommitted in at least *some* fish. The finding that AVT increases around this time reinforces Wong's idea about the start of the sex change window: because both total brain AVT and gsbCYP19A1 aromatase increase concomitantly, it is possible that gonadal events and changes in brain plasticity are converging at this point to influence or direct the process of sex change. However, I emphasize that these only suggest the *beginning* of a sex change window.

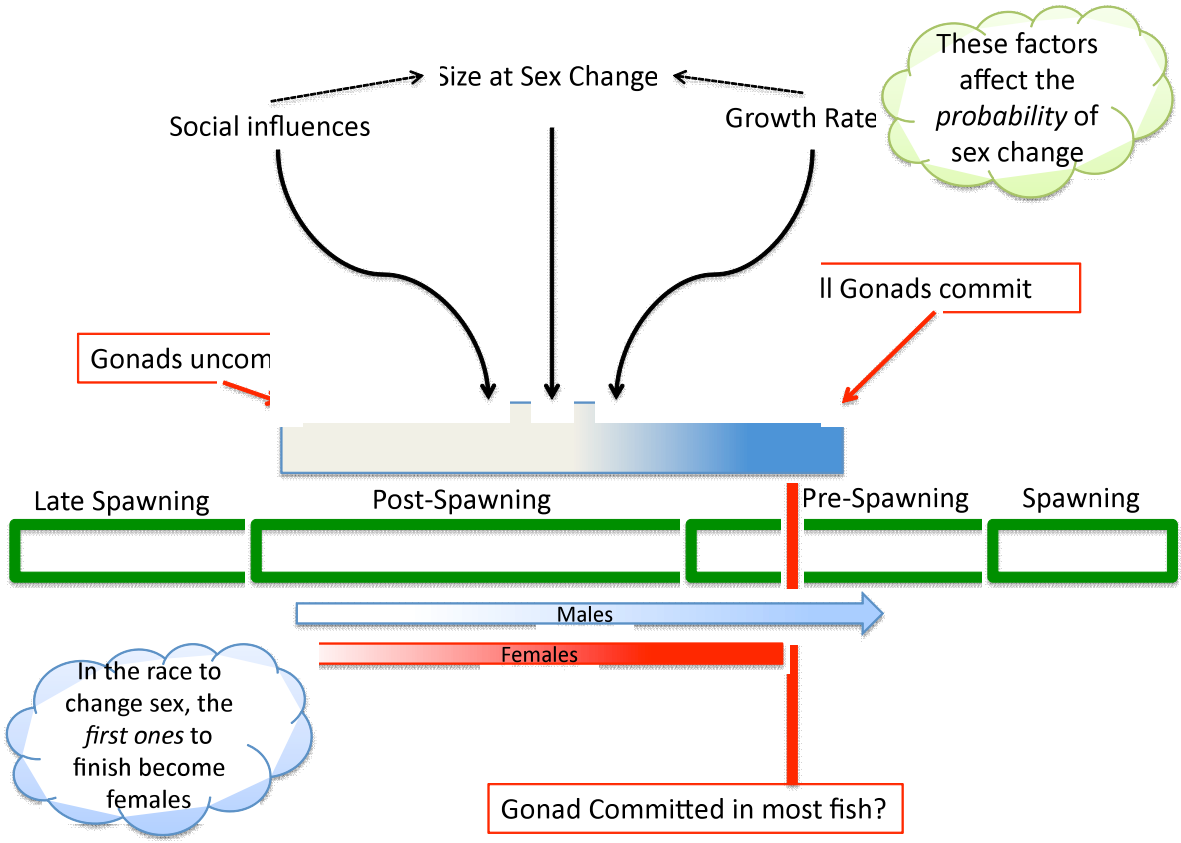


Figure 41. A simplified model that incorporates all the putative exogenous factors that affect the outcome of sex change in the Sparid protandrous hermaphrodite, Gilthead seabream. Note the difference between males and females in the timing of sex change.

Why is the sex change window in these fishes so protracted in comparison to other species? Seabream is an asynchronous spawner with asynchronous gonad development. Hence, not all the fish commit at the same time. Based on gonad histology, it seems that the last to commit are the males (Zohar et al., 1978). Unlike clownfish or wrasses, the extended sex change window in seabream provides for a protracted period where social influences, directly or indirectly, can affect the outcome of sex change. This might be the reason why it is so difficult to narrow a sex change window and why it is so hard to dissect the social influences that can potentially affect

sex change. However, the time window for sex change can only exist for a limited time because the fish that have not committed will need time to prepare for spawning (gonads, sexually dimorphic characters, brain masculinization/feminization, etc.).

The orange opercular color in seabream females peaks in October. In males, intensity of the yellow abdominal color also increases in October, but in females it peaks in January, suggesting its role as spawning advertisement. Because the peak in female orange spot coloration occurs *before* spawning, the only logical explanation is that it serves a purpose other than spawning advertisement. Since the intensity starts increasing around the month of August, it is possible that the coloration plays a role in the advertisement of sex to potential sex changers. Also, it is notable that there is a significant difference between intensity of coloration among males and females. Females seem to have the most intense orange and yellow markings. Males start developing a yellow stripe mark, typical of female fish, but only before females develop their orange spot. Afterwards, males lose the yellow stripes and only females gain coloration in both the yellow and orange markings. Perhaps, males with these yellow stripes in the early month of sex change are attempting to become females, or they are males that might remain sexually plastic until gonad committal occurs. Alternatively, it might happen that these fishes (with the yellow markings) are going to become females, but stop sex change due to some exogenous environmental factor, and begin to commit as males. Because structural colors, such as these iridescent markings, might require days or weeks to revert, it might be that males with these markings are simply intersex individuals. This alternate explanation is most likely, since gonad committal in seabream seems to occur before

October. This explanation cannot completely preclude the concept of a 3- or 4-month sex-change window.

Changes in intensity of the yellow stripe and orange spot start peaking before serum estradiol or serum testosterone increase above basal levels, but just after AVT peaks. Thus, AVT might be involved in such structural color changes, or it might be that AVT is involved in the behavioral response to these color changes. Regardless, the terminal sex of every species in which sexual dimorphisms have been studied, is usually more "colorful" than the initial sex. In this aspect, the Gilthead seabream fits perfectly with the core postulates of Sex Allocation Theory and Charnov (1982) evolutionary explanations of sex change.

Taken together, these data suggest that although the molecular aspects of sex change may have an earlier window, the putative social factors that influence sex change may begin to affect the individuals starting in July and extending to August, but in some individuals (males), to September. The physiological explanation for such a long sex change season is that the seabream have asynchronous gonad development. Hence, the coloration of the more advanced fish, the developing females may indicate to ambisexual males not to change sex. To strengthen this idea, I submit that key neuropeptides that might be involved in the manifestation and transduction of these social signals, such as AVT and GnRH-3, also show significant upregulation during this time period, which is a period of time after the previously proposed window of sensitivity for sex change. I postulate that the sex change window opens around the month of July or August, and does not close until around September, after which the development of the gonad is decided and the males cannot change sex anymore. It is possible that these events take

place during the final segments of the journey that seabream undertake from their feeding grounds in lagoons and near-shore environments to the open seas in which they spawn.

Chapter 4: Sex Change Correlates

Introduction

Although sex change in many teleost fish is mediated by environmental sex determination, the exact cues that trigger or direct sex change are not fully understood. Several mechanisms have been proposed (e.g. Perry and Grober, 2003). In seabream, the contribution that environmental factors may play in the determination of sex is unknown (Happe and Zohar, 1988; Zohar et al., 1984). Indeed, factors such as size of the animal at sex change have both intrinsic and extrinsic determinants. This makes it very difficult to design and interpret experiments involving (presumably) socially-induced hermaphrodites. In this chapter, I explore which factors are more likely to determine sex change in seabream. By testing behavioral, physical, and endocrine parameters, I will attempt to elucidate the contribution of each of these to both sex change and dominance hierarchies. The question of size as the quintessential determinant of sex determination in seabream will be addressed, as well as the relationship between dominance hierarchies and sex change.

In fish, dominance rank is often correlated to various physiological parameters, some of which probably affect the dominance status of the animal, such as growth rate and size (e.g. Montero et al., 2009) , and some of which are a consequence of the dominance rank, such as reproductive success (Dewsbury, 1982; Berejikian et al., 2001). Reproductive success, like dominance, is also affected by size or growth rate (de Gaudemar et al., 2000). This circular relationship also occurs in sex change: If sex change is determined by dominance rank, then any factor that affects dominance rank

could affect sex change. As such, those factors could be construed as being proximate factors of dominance. This is evident in the most reported determinant of sex change in most species, size (Allsop and West, 2003), but from this one should not conclude that either size or dominance is the ultimate (or distal) determinant of sex change. Rather, such evidence only points out to the observable (hence, proximate) cues of sex allocation.

Any factors that affect size, growth rate, or dominance rank, is a candidate factor that could be manipulated to alter sex ratios in seabream. Here I will investigate the correlation between dominance rank and the neuroendocrine factors AVT and the GnRHs, as I did for the gonad cycle and these neuropeptides in **Chapter 3**. To explore this question, several groups of four to six fish were allowed to interact under different social conditions. Based on the knowledge gained from previous chapters, I hypothesize that in this species, dominance rank and size of fish, are the two most important variables that determine sex change. Since the size and growth of the fish are dependent on dominant status, I propose that dominance rank is enough to account for the outcome of sex change.

Methods

Experimental Animals

1. Hexads 2008

The young, 1-year old, ambisexual fish used in this experiment were reared from juveniles at our Aquaculture Research Center facilities in 2.0 m^3 tanks. At the end of the experiment, these fish would be 2 years old. Six months prior to the beginning of the experiment, the animals were placed in a large 4.0 m^3 tank containing older (5+ year old) females. In this tank, the photoperiod, water temperature, and salinity, were manipulated

to slowly advance the spawning period by 6-months. This was done by phase shifting the photoperiod, and by slowly changing the temperature of the tank, to match what the animal would be exposed 6 months later. Similar procedures are used regularly in the aquaculture industry to induce off-season spawning (e.g. Tate and Helfrich, 1998).

Since the rest of this thesis deals with regular photoperiod fish, the dates given correspond to the "equivalent" month once the six-month shift is taken into account (e.g. the sampling that occurred in December is presented here as occurring in the "June equivalent" date).

2. Tetrads 2005

In this chapter, I discuss the results of the neuroendocrine correlates for the experiments described in **Chapter 2**. The description of the animals and the experimental setup used for these tetrads is described in detail in the method section of that chapter. Briefly, two males or ambisexual fishes, and two females or regressed females, were allowed to interact for a period of 8 to 12 days during the spawning stage (2 groups) and the quiescent stage (2 groups) of gonad development. Upon sacrifice, the brains were removed and preserved by freezing them at -80 ^{o}C over dry ice. Later, total mRNA was extracted from each brain using a Phenol extraction protocol described in **Chapter 3**.

Experimental Setup

Originally, six fish were placed in each of the four tanks. The four tanks are physically identical, with the exception of ceiling clearance, which varies with the exact location of the tank. These tanks are divided into a pair of connected systems (6-11/6-12; 6-17/6-18). Each connected system shares filtration media, protein skimmers, water temperature control, main recirculation pump, etc. Three large females (5+ year old) and three males

(1 year old) were placed in each tank, except in 6-17 were only one female was placed with five males. Due to problems with the anesthetic, several of the females died during or immediately after transferring and tagging them. Furthermore, between Sampling #2 and Sampling #3, all fish in Tank 6-12 and 6-11 died due to failure of the main recirculation pump. I introduced new fish to tank 6-12 but not 6-11, after filming in that tank had proven difficult due to inadequate clearance between the water and the camera.

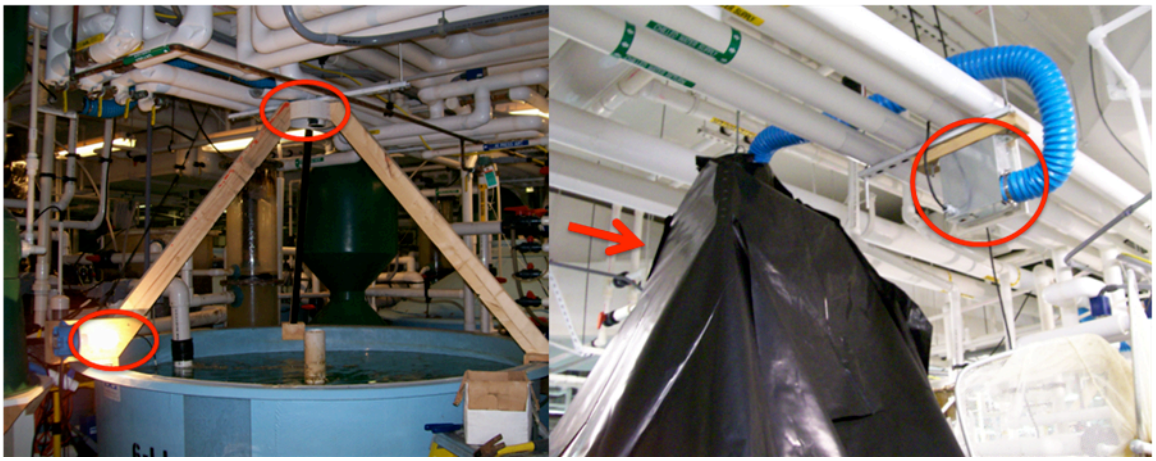


Figure 42. *Teepee-like support structure for the camera (left picture, top circle). The lights on this particular setup are on the side (left picture, bottom circle). A black plastic sheet was used to cover the teepee after it was built (right picture, arrow). A ventilation system with a fan (right picture, circle) was built in systems that had lights on the top, to prevent the camera from overheating.*

Recording of Behaviors

Each 2.0 m^3 tank was modified by removing the cover and placing a large triangular, teepee-like, custom cover on top of the tank (**Figure 42**). Inside the top of this cover a camera was placed, pointing downwards towards the surface of the water, so that the fish could be filmed from a top-down view. Since these animals are on a strict photoperiod, each side of this triangular structure was covered with black plastic sheeting. This sheeting did not allow a significant amount of ambient light to enter the tank and prevented the animals from being disturbed by the daily activities of our staff. The cover

also had 3 lights, placed on the bottom (6-18 and 6-11) or the top (6-17 and 6-12) of the support beams. A feeding tube was provided and a filming schedule was posted outside the tank to minimize disturbances during filming of the animals. After a fish jumped out of the tank to its death, plastic rings were placed to hold the black sheet covering firmly in place.

Video was recorded as explained in previous chapters. The four BNC cameras were connected to a single Winnov Video Capture card. A custom program, created using Visual Basic and Winnov's SDK, recorded 15 minutes of video at preset interval, cycling between 2 or 4 camera sources and automatically naming the file so that the source camera, tank name, date and time of recording, and capture rate, were included in the file name. Recording took place 3 times a day in each tank, usually around 12:30pm, 2:30pm, and 4:30pm.

Approximately 60 hours of recording was carefully analyzed, consisting of various spawning and quiescent sampling periods. This represented a total of 240 files. All data was processed using *AquaObserver* as described in **Chapter 1** and **2**.

Sampling and Tissue Collection

Each sampling occurred approximately every four to six weeks for a total of 9 sampling periods. The day of the sampling, the tanks were drained to approximately 50% of their regular water level. The main pumps were switched off then anesthetic (clove oil or MS-222) was placed in the water while carefully monitoring the fish for signs of stress. When the fish were showing signs of sedation, they were carefully transferred to a small 15g container with MS-222 or clove oil. This anesthesia tank also contained a single airstone. While clove oil has been shown to be safe for seabream (Mylonas et al.,

2005; Bressler and Ron, 2004), the older females (5+ years) did not fare well on this anesthetic and after sampling #3, I switched to MS-222. MS-222 is a very safe sedative and anesthetic, and the only one approved for food-grade aquaculture.

When the fish was in deep anesthesia, I collected a 1.0 ml blood sample from the caudal vein, using an 18 gauge needle and a heparinized 1.0 ml syringe. The blood sample was placed on ice immediately. The fish was removed from the water and placed in a scale to measure its total length. In the first sampling, this was followed by placing a PIT tag on the fish dorsolateral area. From then on, I placed the animal on a surgery table, read the PIT tag, and based on that tag, I selected an appropriate color bead as a visual tag. I placed one color bead in each side of a fish dorsal fin, using a procedure developed by COMB's aquatic veterinarian, Dr. Brent Whitaker. The tags were tied to, and placed between, the rays of the fish using Polyethicon™ monofilament. Because the surgery was done out of the water, care was taken that the fish would be returned to the water as quickly as possible. The recovery of the fish was also closely monitored. When all the fish in one tank were processed, the main pump was switched on, another 25% of the tank volume was emptied, and new salt water and fresh water were used to bring the tank back to 100%. This was done to eliminate the sedative effects of the anesthetic by simple dilution. Each tank system is equipped with an ozone-injected protein skimmer, which can get rid of any MS-222 or clove oil remaining in the water, in the following 24 hours. Filming was commenced the next day, once all traces from anesthetic had been eliminated from the fish and the tank water.

Gene and Hormone Quantification

The assays to quantify gene expression for AVT, and the 3 GnRHs, and the

Radioimmunoassay used to quantify estradiol and testosterone are described in Chapter 3.

Statistics

Parametric methods were used where appropriate, but most of the data reported was non-parametric (ranks). Spearman rank correlation test were used and results only reported as significant if below the 0.05 level. Trends were reported if the significance was closer to the 0.10 level. Most tests were ran in Graphpad Prism, but JMP was used for a multiple linear regression model for correlating AVT, and the 3 GnRHs, to rank. If the number of individuals in a "treatment" group was less than 3, any difference was reported as insignificant.

Results

1. Hexads 2008

Sex Change

Some fish were lost before they could have completed sex change. Only Tank 6-17 contained two males (#0D41 and #536C in **Table 6**) that did not complete sex change. At the time of sacrifice, during late spawning, the gonad is no longer ambisexual although it is still bipotential in the males (see **Figure 1** in the **General Introduction**). Thus, at this point in time, the fish are either male (with the potential to change sex next year) or terminal female. Sex was confirmed by histological sectioning and staining of gonad tissue.

Tank/Fish ID	SEX	Sampling Date (Equivalent if not 6-mo Advance)								
		14-Feb-07	14-Mar-07	19-Apr-07	28-May-07	26-Jun-07	23-Aug-07	23-Sep-07	5-Oct-07	19-Nov-07
6-12										
6441	M	n/a	n/a	n/a	n/a	37	36	39	39	n/a
6970	M	n/a	n/a	n/a	n/a	39.5	40	43	42	n/a
316A	M	n/a	n/a	n/a	n/a	36	38	39	40.5	n/a
226A	M	n/a	n/a	n/a	n/a	35	DEAD	DEAD	DEAD	DEAD
177A	M	n/a	n/a	n/a	n/a	38	38.5	40	40	n/a
4E0F	M	n/a	n/a	n/a	n/a	37	39	39	39	n/a
6-17		14-Feb-07	14-Mar-07	19-Apr-07	28-May-07	26-Jun-07	23-Aug-07	23-Sep-07	5-Oct-07	19-Nov-07
000A	M	38	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
1940	F	58	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD
0D41	M	40	41	41	42	n/a	42	43	45	n/a
3B1E	M	40	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD
263B	M	n/a	41	42	42	n/a	46.5	46	46	n/a
536C	M	n/a	34	35	36.5	n/a	36	37	37	n/a
654A	M	n/a	37	37.5	40	n/a	40	41	40	n/a
617A	M	n/a	41	41	40	n/a	42	43	42	n/a
		14-Feb-07	14-Mar-07	19-Apr-07	28-May-07	26-Jun-07	23-Aug-07	23-Sep-07	5-Oct-07	19-Nov-07
6-18										
7D57	F	53	54	54	54	n/a	53	55	55	n/a
400A	M	32	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD
287D	M	36	35	38	37	n/a	38	39	39	n/a
4235	M	38	37	40	40	n/a	40.5	42.5	41	n/a
567A	F	58	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD
7D1F	F	59	58	58	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD

Table 6. Sex and PIT ID of each fish used in the experiment. Length of animals is given as distance between the tip of the snout and the end of the caudal fin in centimeters. Sampling date is given as the equivalent, since fish are in a 6-month advance photoperiod.

The sex change rate for each tank was as follows: 6-12 had 100% sex change plus one fish that died before the final sampling, 6-17 had 67% sex change plus one fish that died very early in the experiment, 6-18 had 100% sex change, with various fish deaths mostly at the beginning of the experiment.

Tank 6-18 was the only tank in which a large and older female, introduced to suppress sex change at the beginning of the experiment, survived. This female was sick, perhaps from anesthesia-related kidney or liver failure. Upon sacrifice, her gonads were discovered to be in a severe pathologic state, with very loose tissue and a lack of

vascularity in the gonad. However, histology of the gonad showed some pre-vitellogenic and vitellogenic oocytes, as well as many atretic oocytes. A fish from tank 6-17 died just before the end of the experiment (PIT #654A). However, analysis of the blood samples obtained from this fish suggests it was a female.

Sex	**Size (cm)**	**Rank during Sex Change Window**	**Growth Rate (cm/mo)**
Male	36	2	0.22
Female	41	1	0.11
Presumed Female	41	4	0.43
Male	44	3	0.33
Female	46	5	0.56

Table 7. Fish from tank 6-17 after sex change. Size is at sacrifice (December equivalent). Growth rate calculated from first and last size measurement. Dominance rank reported is that of last observation period. Note that neither size, nor growth rate can solely account for sex change.

PIT	**SEX**	Rank	**Rank**	Rank	Sex
		March (LS)	**May (Q)**	December (S)	
0D41	M	4	**3**	4	M
3B1E	M				
263B	M	2	**5**	5	F
536C	M	4	**2**	1	M
654A	M	1	**4**	3	PF
617A	M	3	**1**	1	F

Table 8. Fish from tank 6-17 showing rank during different gonad stages. LS=late spawning (before sex change); Q=Quiescent (Sex Change); S=Spawning (After Sex change). 1=Dominant; 5=Submissive. Closer to the initial time of group fusion, rank and sex change outcome seem to correlate as expected.

Body Size, Dominance Rank, Growth Rate and the outcome of Sex Change

Only two males did not change sex, therefore it was difficult to apply any statistic test or to generalize anything from the correlation between sex and rank or size. However, the *trend* was for fish that did not change sex (males) to be smaller and sex changers (females) were bigger (**Table 7**). Similarly, growth rates (cm/month) of males tended to be lower than females, but some females had slow growth rate and yet they changed sex (**Table 7**). Interestingly, the smallest fish in the tank, which stayed as male, was the smallest when it was introduced into the tank.

Rank and sex change outcome did not seem to correlate as expected, but the number of males made it difficult to draw any major conclusions. However the rank of the fish immediately following group formation, seem to follow the outcome of sex change (**Table 8**). As expected from the size-advantage hypothesis (SAH), future females ranked as more dominant than future males. The exact significance of this is unknown as this occurred very close to the end of the spawning season and the beginning of quiescence.

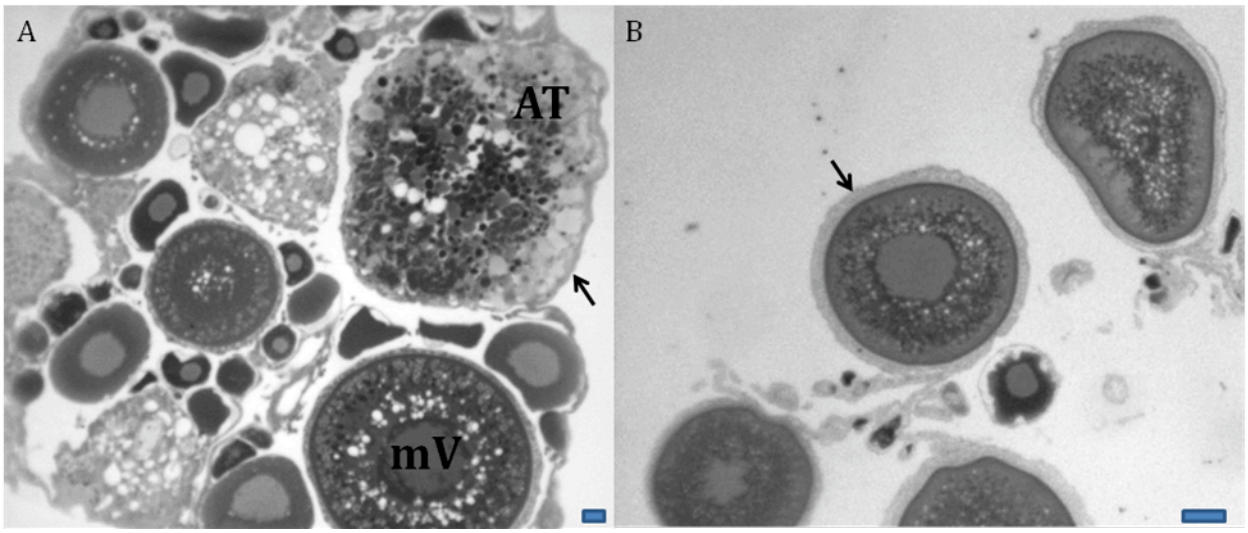


Figure 43. *Histological sections of seabream ovaries.* ***(A)*** *Female without males. Note that although there are a number of mid-vitellogenic oocytes (mV), at least some oocytes are undergoing atresia (AT). The arrow points to the zona radiata, which has broken up already in this oocyte.* ***(B)*** *Female under the presence of males. The oocytes have an intact zona radiata (arrow), and no atresia can be observed.*

Oocyte Development

Atretic oocytes were observed in all the groups which lacked males. In these females, many of the oocytes had undergone complete atresia, with total loss of the *zona radiata* as described by Meiri et al. (2002). Also, consistent with these findings, a significant amount of cellular debris was observed in these sections (**Figure 43A**). In contrast, few atretic oocytes were observed in the tank were males had remained after sex change (**Figure 43B**). Pre-vitellogenic and mid-vitellogenic oocytes were present in most of the females and were observed in the three groups of fish. No oocytes were observed in which migration of the germinal vesicle (GV) had occurred or was about to occur. The migration occurs approximately one or two days before the female is able to release the eggs into the water.

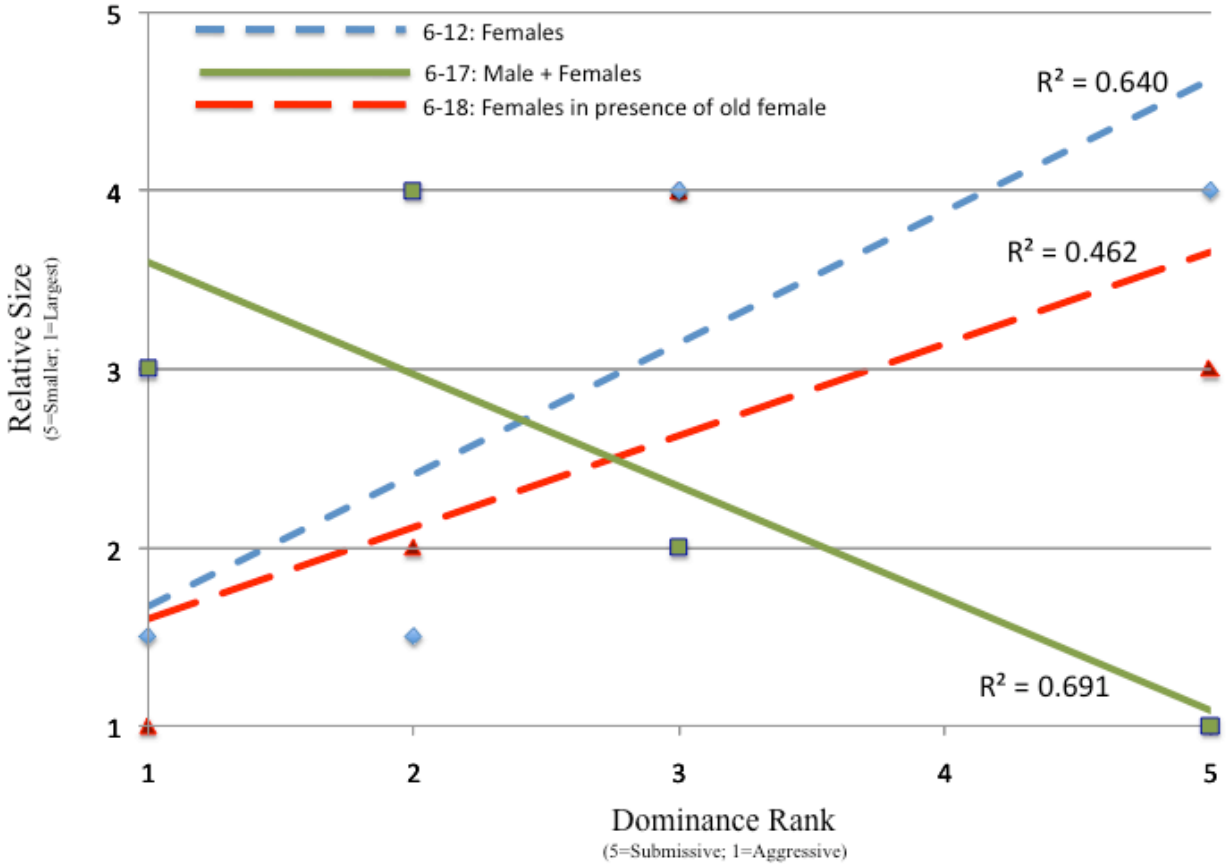


Figure 44. The correlation between dominance rank and size (relative size) for each of the three groups in the hexad experiment. Note that the group in which some males did not change sex (6-17) shows a negative correlation between dominance rank and size. Thus, in this group, smaller individuals were the most dominant.

Dominance Rank vs. Relative Size

The fishes in each group were ranked according to total length, with the longest animal given a rank of 1 (one) and the second longest animal a rank of 2 (two) and so forth. If the animals had the same length, the rank was calculated using the same methodology described in **Chapter 1** for dominance rank calculations involving rank ties. Thus, the dominance rank could be correlated to the size rank. *AquaObserver* gives the most dominant animal a rank of 1 (one). If the biggest animal is also the most dominant, a positive correlation should emerge between the two variables. The result of this correlation for each of the three groups is shown in **Figure 44**. While 6-12 and 6-18,

were both dominated by the biggest fish, 6-17 was dominated by the smallest individual. In fact, the two smallest fish in this group had the highest rank: one was a female and the other was a male. The dataset was too small for each tank (N=4 or N=5). Combining the all-female tanks (6-12 and 6-18) yields a significant correlation (r^2=0.58; p=0.028). Thus, size is positively correlated to rank (**Figure 44, dashed lines**). In the tank in which sex change was suppressed in some individuals (6-17), the correlation was not positive (**Figure 44, solid line**). This correlation showed a trend but it was not significant (r^2=0.64; p=0.104), perhaps due to small sample size. However, a similar observation was made as an interesting anomaly in the tetrad experiments of **Chapter 4**. Thus, it seems that smaller males are more behaviorally active and aggressive than females.

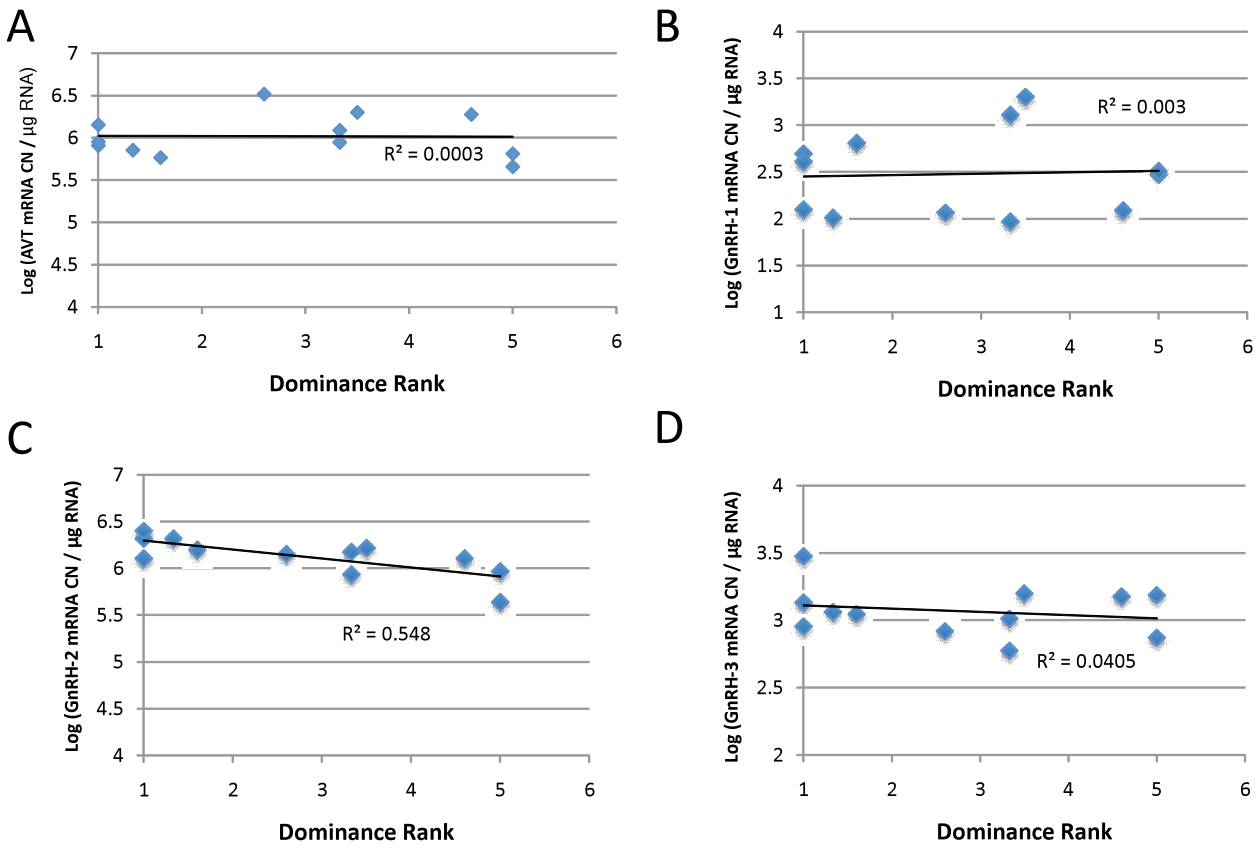


Figure 45. *Quantification of mRNA expression in whole brain of the seabream from the 2008 experiment, correlated to rank of individual using average rank for weeks preceding final sacrifice (December equivalent).* (A) *AVT mRNA shows no correlation with rank.* (B) *GnRH-1 also shows a very weak correlation with dominance rank.* (C) *GnRH-2 shows a very strong and significant correlation ($p<<0.01$) with dominance rank.* (D) *The correlation between GnRH-3 and dominance rank seems to be weak.*

Neuroendocrine Factors

AVT did not correlate well with rank (**Figure 45A**). Of the 3 GnRH isoforms, only GnRH-2 correlated well with fish rank (**Figure 45B, 45C and 45D**). Using a simple linear regression test for GnRH-2 versus average dominance rank resulted in an r^2=0.54 ($p<<0.01$). GnRH-1, GnRH-3 and AVT all had very low r^2 values with very high *p* values ($p>>0.95$).

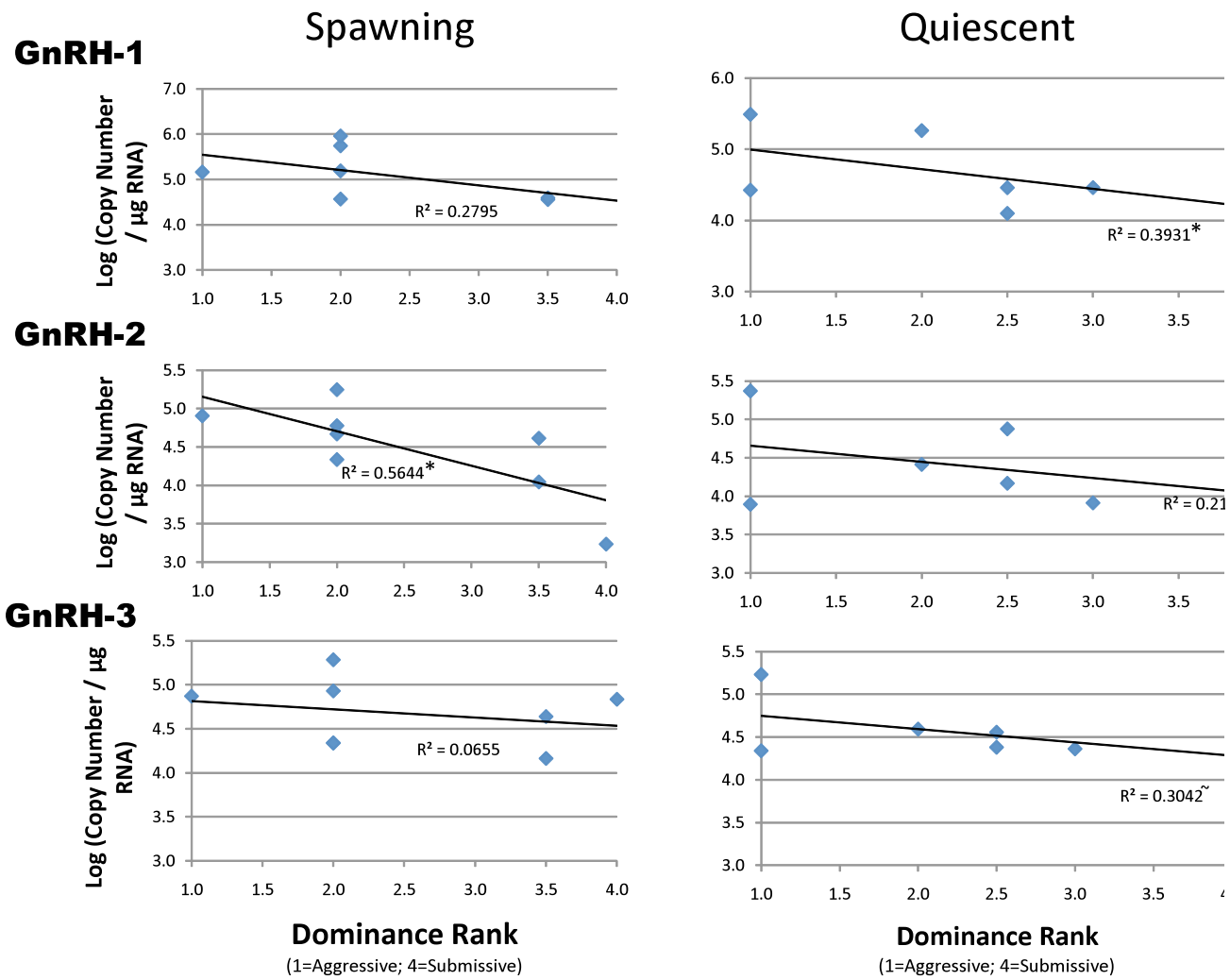


Figure 46. Correlation between dominance rank and the mRNA of the 3 GnRH isoforms of seabream brain in Spawning (N=8) or Quiescent (N=8) tetrads from 2005 experiment. The R square values are reported for each comparison. A Multiple Correlation analysis using least square means was run to report statistical significance (*$p<0.05$; ~$p\leq0.10$). Note that in quiescent fish, GnRH-1 correlates significantly with dominance rank and in spawning fish, GnRH-2 correlates significantly with rank.

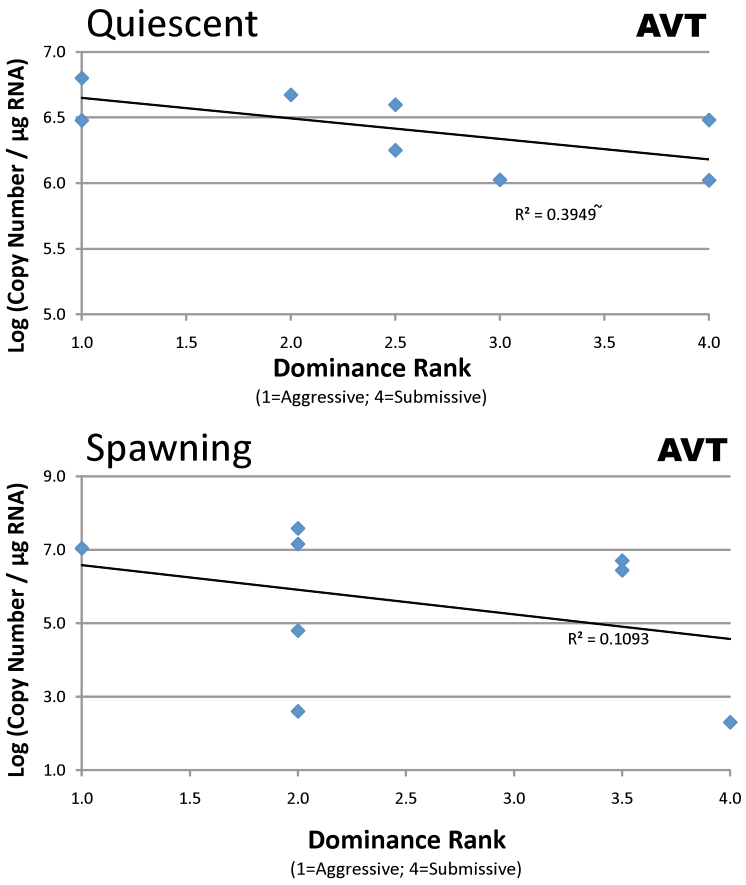


Figure 47. Correlation between dominance rank and whole brain AVT mRNA in Spawning (N=8) or Quiescent (N=8) tetrads from 2005 experiment. The R square values are reported for each comparison. A Multiple Correlation analysis using least square means was run to report statistical significance (**=p<0.05; ~=p≤0.10*), which was run together with the GnRH data.

2. Tetrads 2005

Neuroendocrine Factors

The individuals in the behavioral experiment reported in **Chapter 2** were euthanized at the end of each observation run. After euthanasia, the brains were extracted and placed over dry ice. The RNA was then extracted from each brain, following the protocol described in **Chapter 3**. Using RT-PCR, the amount of mRNA for each of the neuropeptides of interest was quantified. The amount of mRNA was then correlated to

the median dominance rank of each individual in the group. The correlation was analyzed using a multiple regression analysis model based on least square means (**Figure 46** and **47**). The quiescent and spawning group each consisted of two separate tetrads, for a total of eight fish in each group. The statistical program used in this analysis adjusted the correlation coefficient to account for the fact that each group observed (i.e. tank) has different average hormone levels. Thus, the model accounted for that source of variance. The correlation coefficient reported in the text, differs from the figures because of this reason.

In spawning groups GnRH-1 did not correlate well with rank (r^2=0.79; p=0.25); GnRH-2 showed a significant relationship with dominance rank, so that the more dominant the individual, the higher the levels of GnRH-2 (r^2=0.22; p=0.04*); GnRH-3 did not show any correlation to dominance (r^2=0.22; p=0.52); As with the 2008 experiments, AVT did not show a relationship with rank (r^2=0.02; p=0.44). In quiescent groups GnRH-1 had a strong and significant relationship to rank, so that the more dominant an individual was, the higher their GnRH-1 expression. As opposed to what was observed in the spawning stage, GnRH-2 was not correlated to rank (r^2=0.04; p=0.28). However, like in the spawning stage, GnRH-3 (r^2=0.58; p=0.10) and AVT (r^2=0.35; p=0.10) expression did not correlate significantly with rank.

Discussion

Since only two males did not change sex, it is difficult to draw any major conclusions about the effects of some of these variables on sex change. However, some trends are observed (**Table 2**). Unlike what was expected, there was no significant correlation between AVT and rank in this experiment. Of the 3 GnRH isoforms that were assayed, only total brain GnRH-2 showed a strong correlation to calculated rank in the 2008 experiments. Recall that, as shown in **Chapter 3**, the gonad cycle affects the expression levels of these four neuropeptides, and these fish were euthanized in the month of December (the equivalent date in this 6-month advanced population). This corresponds to the spawning stage of gonad development. Thus, the spawning groups from the 2005 tetrads also showed a significant correlation with dominance rank only with GnRH-2. In the quiescent groups from these tetrads, GnRH-2 and dominance did not show a correlation. Instead, GnRH-1 showed a strong correlation to dominance rank. Meanwhile, GnRH-1 was significantly correlated to rank only during the quiescent stage of gonad development. These results are similar to what has been observed in cichlids under social stress (Ogawa et al., 2003).

What is the relevance of a significant correlation between GnRH-1 and rank during the quiescent period? GnRH-1 is the hypophysiotropic form in seabream (Powell et al., 1984). It is probably involved in directing or initiating gonad development (Parhar et al., 2003). Under the assumption that sex change is a race (see **Chapter 3**), it is possible that the first factor to influence sex change is dominance: dominant fish begin this race first. These fish become females and reach gonad commitment before other fish can. Fish that do not reach gonad commitment at some hypothetical point-of-no-return,

would then "turn around" the gonad reversal and become males. The model is partially supported by the fact that rank and the outcome of sex change showed a trend that is consitent with this model: fish that became males were less dominant, around the time of sex reversal.

In the 2008 experiment, the correlation between GnRH-2 and dominance was positive, so that the most dominant fish had the highest expression levels of GnRH-2. Ogawa et al (2003) showed that social stress downregulates only GnRH-2 and GnRH-1 (but not GnRH-3) expression in tilapia. Subordination increases social stress in seabream (Montero et al., 2009), so that those individuals that are under the dominance of others have higher cortisol levels and depressed immunity. Tilapia is a gonochore, and therefore the gonads do not exhibit the protracted quiescent (and ambisexual) gonad stage observed in Sparids, which might explain the season effect seen in seabream but not in tilapia. Thus, the gonad cycle directly influences this relationship. That such an effect could be due to the actions of gonad steroids should not be overlooked, considering that, for example, Klenke and Zohar (*in press*) have shown a similar effect on GnRH-2 in stripped bass during different stages of gonad development. In both *in vivo* and *in vitro* studies, they showed that the effect was due to downregulation of GnRH-2 by estradiol.

As expected, dominance rank and size were strongly correlated (in tank 6-12 and 6-18; the "all-female" tanks). This is consistent with what I observed in the dyadic encounters between sex-matched individuals (**Chapter 2**), but not with the observation of the tetrads (**Chapter 2**). However, both the 2005 tetrads and tank 6-17 from the 2008 experiment show a tendency for smaller fish to dominate over bigger fish. It is possible

that such a relationship is related to the above stated possible mechanism for sex change, but more research is needed to understand this paradox.

Although there is wide variation in the expected sex change ratios, most reports seem to suggest that in two-year old captive seabream the sex change rate is 80% (e.g Happe and Zohar, 1988). Therefore, we expected the control group to have at least one male, while in the groups in which older females were present, it was expected that fish would not change sex. Out of a total of 12 possible fish that could change sex, 10 fish became females (82%). Thus, assuming no tank effects, the fish changed sex at a normal ratio. However, the two males that did not change sex were in one tank, and the previously reported effect of sex change suppression in the presence of older fish, did not occur. There are various possible scenarios that can account for this unexpected result.

Tank 6-18 had several females during the sex change period. However, some of these females were lost before sex change. In this tank, only one older female remained at the end of the experiment. This female had an underlying pathology (see the **Methods** section). However, considering that in this fish, the gonad was able to produce some steroids (data not shown) and oocytes, it is reasonable to assume that this fish would be able to suppress sex change in the remaining fish. However, the one factor that distinguishes this female from the others in this group is that this female was behaviorally suppressed (data not shown). The behavioral suppression of this female somehow translated into the suppression of the entire group, as can be observed from the rank changes that occurred between the 4^{th} and 7^{th} observation period.

Taken as an individual group, tank 6-12 had a sex change rate of 100%. Fish in this tank were replaced with a new batch of fish from the same source tank because of the

accidental death of all the animals around the 4th sampling period. Replacement occurred in the 5th sampling period (**Table 6**). This sampling date corresponds to the equivalent of late June. Thus, this transfer occurred *after* the **beginning** of the sex change window, and just about one or two months prior to its hypothesized closure. To recap, the sex change window seems to open around June (Wong, 2006) and closes around August (**Chapter 3**). It is possible that I inadvertently transferred the most advanced sex changing individuals to this tank. Such females do not retain the capacity to reverse sex, as gonadal committal has already occurred. The probability of this occurring is low, but given the small number of fish transferred, it is a possibility that should be considered.

Considering that in this species, sex change occurs in isolation at various ratios (Wong, unpublished) and that sex change does not seem to follow a strict size-threshold rule, at least *in captivity* (see **General Introduction**), it would suggest that either sex change occurs randomly or that it is affected by more than one factor. Sex change is affected by the presence of older and bigger females (Happe and Zohar, 1988), thus it follows that it is not a random process. Grober and Rodgers (2008) point out to the "overreliance" on size as a cue in the analysis of sex change, stating that: "…over-reliance on reductive cues like size… has constrained our thinking about the evolution of sexual plasticity and supports the continued publication of papers… that disregard… how animals make decisions about both 'gender role' and sexual allocation.". It seems that the persistent elusiveness of the putative factor that controls sex ratios in seabream might be proof-in-point of the overreliance on size and age as proximate factors that account for the decision to change sex. Neither ESTM nor SAH (see **General Introduction**) can account for the data presented here.

In conclusion, the results presented here do not provide support for the hypothesis that dominance rank is the *only* determinant of sex in seabream. The significance of this relationship could not be assessed but it seems that the *trend* was for future males to be less dominant around the time period just before sex change, while most future females tended to be more dominant than future males, and this was consistent among different observation times. The existence of olfactory cues cannot be discounted. The experiments provide support for a role for GnRH-2 and GnRH-1 in dominance hierarchies. The existence of a quiescent-only correlation between GnRH-1 and dominance rank suggests a mechanism by which activation of gonad maturation could occur earlier in dominant fish. Further research would be needed to assess the exact role of GnRH-1 in sex change, the role of GnRH-2 in dominance during spawning, and how dominance might be involved in the decision to change sex.

General Conclusions

This work set out the goal to understand the interplay between behavior and sex change in the Gilthead seabream. To progress towards this goal, the behavior of seabream had to be studied. There was very little information in the literature concerning the captive or wild behavior of seabream, other than multiple reports of spawning or migration in the open seas. The recent work by Montero and coworkers (2009) is one of the very few published works on sea bream captive behavior. The scarcity of this work in the literature is perhaps due to the perception that animals such as seabream do not display the wide range of behaviors observed in more popular fish models (e.g. clownfishes, cichlids, wrasses, and gobies), and due to the difficulty of observing, and visually identifying, large fish. This study overcame many of these difficulties by developing a set of tools to quantify behavior. The toolbox for quantifying seabream behavior is contained in *AquaObserver* and can be used to quantify behavior in other species as well.

Using *AquaObserver*, I was able to study seabream behavior in a way that was never done before. Careful observation of the animals led to the discovery color changes that occur year round in the fish (**Chapter 1**). Using the Image Analysis tool of *AquaObserver*, I was able to quantify the speed at which the animal changes color. The fast color changes could play a role in the spawning behavior, but they also seem to play other roles, such as the defense of territory (**Chapter 1**).

Seabream behavior had never been characterized before in regards to their behavioral repertoire. Using Markov Chain analysis, I was able to present each of the

behaviors exhibited by this fish in the context of how, and when, the animal displays the behavior. This analysis allowed me to answer some basic questions about seabream behavior. I found that color changes represent the escalation of aggression, and that the intensity of aggression bouts increases during the quiescent gonad stage. Another change associated with gonad cycling is that the individuals loosen their hierarchical association, which is very linear and stable during the quiescent stage of the gonad cycle. Such effects have never been explored in seabream. The results from this experiment, and the lessons learned from some of its shortcomings, also proof useful in helping to design a better experiment to test the relationship between dominance and sex change.

At the core of this project was also the question of how dominance is transduced into a sex change determinant. Extensive work by various researchers around the late 1990's and early 2000's had shown that AVT in the preoptic area of hermaphrodite fish was strongly correlated to sex change (Godwin et al., 2000; Grober and Sunobe, 1996). It was hypothesized that AVT had a role in sex change (Foran and Bass, 1998), but little was known about how this neuroendocrine factor interacted with the GnRH system, and if it changed as a consequence of sex change or if it played a regulatory role. Thus, I wanted to find out if AVT was correlated to sex, and if it played any role in sex change.

Early in the development of this project, I was able to clone the complete mRNA for brain AVT in seabream. To this date, this is the only report (still unpublished) of cloning of AVT in seabream. Using anti-AVP from a mammalian source, which had already been shown to cross-react with fish AVT, I was able to show where these cells are located in seabream. To further study AVT expression in the brain, I developed an RT-PCR assay. There was a trend for females to have higher AVT expression levels than

males. However, this was not statistically significant and will require further experimentation.

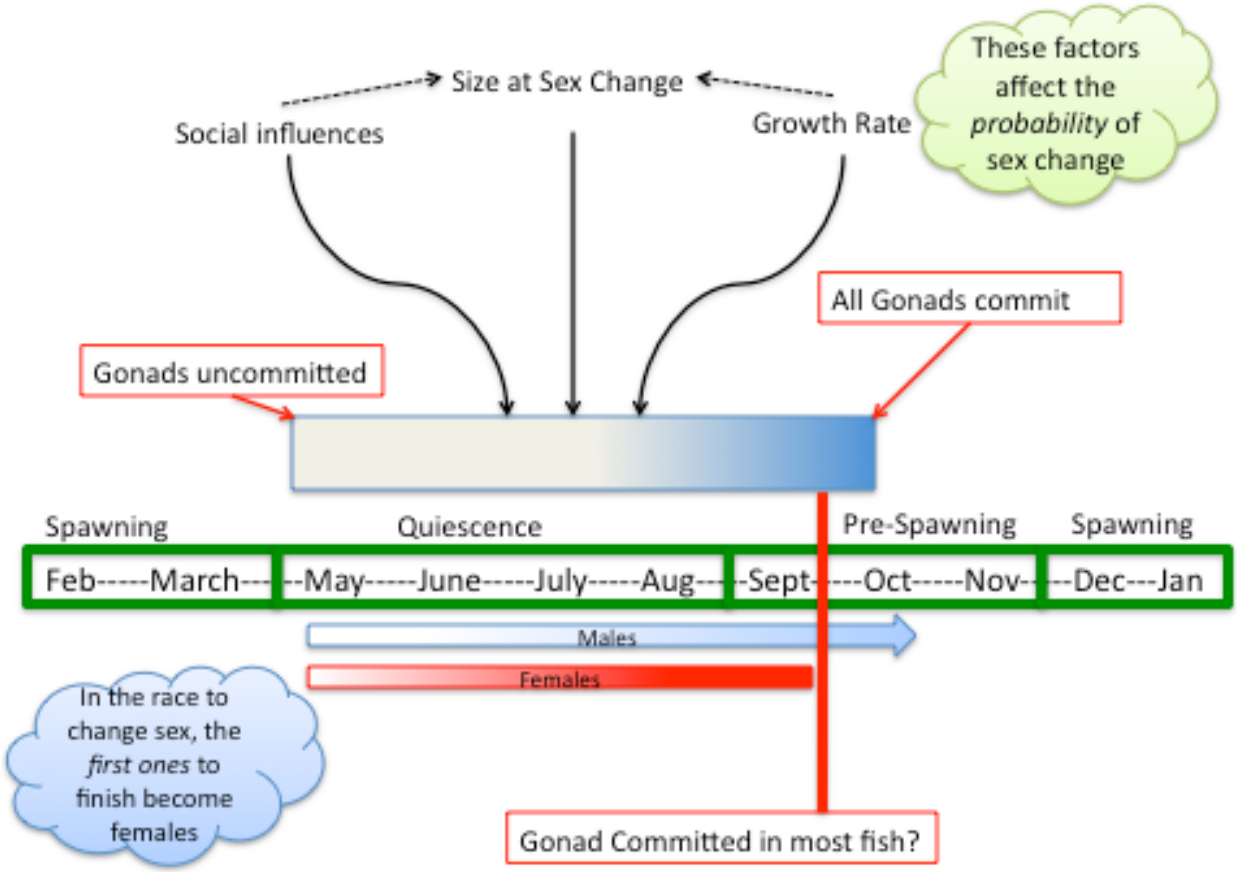


Figure 48. Gonad development stages in seabream and the different behavioral, physiological, and neuroendocrine, events that co-occur with the development of the gonad.

Sex Change: Sequences of Events in the Gilthead Seabream

The sequence of events that surround the period of sex change and gonad development are shown in **Figure 48**. Wong (2006) reported an increase in gonadal aromatase during the month of June, and here I report an increase in AVT during July. Thus, we first see an increase in gonadal aromatase, followed by an increase in brain AVT. Are these events connected? Further research is needed to answer this question,

but AVT cells containing estrogen receptors have been found in the Atlantic croacker and a possible connection between brain aromatase and AVT has also been shown in wrasses (Review: Godwin, 2009).

AVT also increased again, together with all the GnRH's, during the spawning period. AVT plays important and highly specific roles in certain behaviors related to spawning and reproduction (e.g. Salek et al., 2002). In seabream, this increase in AVT levels is preceded by an increase in coloration in females and the males. This experiment was not designed to test the direct relationship between these events and any of the neuroendocrine factors assayed.

The expression of the 3 GnRH isoforms was studied using quantitative RT-PCR. The seasonal profile of GnRH expression was studied. GnRH-3 levels increase in July, just before AVT levels increase (in August). The expression of all 3 GnRH's increased in the month of December. GnRH-3 and AVT were strongly and significantly correlated, a fact that seems to suggest that there might be a relationship between the peak of GnRH-3 and the posterior peak in AVT. GnRH-3 has been shown to be involved in the modulation of olfactory and visual information in the context of reproduction, and it is possible that this peak is related to this role of GnRH-3.

This is the first report of the correlation between dominance and sex change in seabream, and the first report of a correlation between a GnRH and dominance in this species. Due to the small number of males remaining in the group after the round of sex reversal, it was not possible to draw any significant conclusions. However, it seems that being a dominant fish was important to the decision of changing sex. Neither size nor dominance could completely account for the decision to become male or female. It

seems that the connection between intrinsic and extrinsic factors that affect sex change is much more complex. For example, growth rate affect both size and energy reserves. Size has been previously implicated as a factor in seabream sex change (Chaoi et al., 2006; Happe and Zohar, 1988; also see **General Introduction**) and energy reserves availability is an integral part of the size-advantage hypothesis of sex allocation theory. Growth rate is the product of whole body metabolism (Berne and Levy, 1998), and as such can be expected to be under the influence of many genes. Regardless, growth rate will also be affected by the position of the animal in the hierarchy because this position also affects the animal's access to resources (Wilson, 1975).

Alternate View of Sex Change

A size or age threshold has been proposed for various sex-changing species (Allsop and West), including seabream (Chaoui et al. 2006). Size is also an important determinant of dominance hierarchies in many hermaphroditic species (Fricke and Fricke, 1977). Dominance hierarchies follow sex changing "hierarchies" in wrasses and clownfish (e.g. Godwin et al., 1996). However, not all fish seem to follow the "size-advantage" hypothesis (SAH), which is one reason why Munoz and Warner (2004) proposed the "expected reproductive success threshold" (ESTM) hypothesis, as an alternate. Reports of sex change in isolation (e.g. Carruth, 2000), and sex change after removal of males or females from a group (e.g. Fricke and Fricke, 1977), suggest that the size threshold, if it exists, is a context-dependent variable. Indeed, the reliance on size (or age) as a sex change cue, seems to support the idea that such proximate factors can, by themselves, account for the internal physiological state which trigger sex change or determine sex itself (e.g. Allsop and West, 2003; Munoz and Warner, 2004). Indeed, it

seems that researchers continually assume that any observable criteria (e.g. size or dominance) accounts can be linked to the ultimate factors that determine sex change.

Another possibility is that because SAH assumes equal growth rate among males and females, then one reason why some species, such as seabream, do not follow a strict version of SAH is because growth rates differ between males, females, ambisexual juveniles, and older fish. Sex change ratios reported in the **Chapter 4** 2008 experiment and the size distribution reported in the **General Introduction**, support the idea that in seabream sex change is not completely dependent on size. That dominance might still play an important role should not be completely discounted, considering the results reported here (**Chapter 4**). Also, SAH is usually seen as relying on ultimate cues to explain the allocation of resources between males and females, SAH might be true in most cases only because size is in itself the most conspicuous, yet proximate, factor: An individual's size may simply reflect the convergence of many physiological and behavioral parameters (e.g. energy, motivation, ability to fight and control resources), but it does not stand alone as the ultimate factor that determines sex allocation. Also, as mentioned above, size in turns affects dominance status, growth rate, and might even affect the speed of gonad development, which adds to the complexity of the system. In a situation where these factors do not interact or behave in the same way for each sex (e.g. males grow faster than females), under different environments (e.g. a small male in the presence of females vs. in the presence of males), or if the relationship between them were not linear (e.g. smaller fish attain dominance faster), then the resulting skew would affect the relationship between sex and size, perhaps in ways that are difficult to predict.

Recently, Montero et al. (2009) showed that seabream form linear hierarchies and that those fish at the top of the hierarchy eat more and grow faster. Thus, neither cause and effect, nor intrinsic versus extrinsic, can be dismissed easily as confounding factors in this equation. The consequence is that dominance hierarchies, growth rate, and fish size form an inextricable chain of cause and effect, on which the decision to change sex probably depens. In fact, more likely such a decision occurs as the sum of this hypothetical chain converges into some determining threshold. The weight of each of these factors is unknown but the complexity of such system could account for the fact that the answer to the question of how sex ratios are regulated in seabream has remained so elusive. At the very least, it seems that size and dominance are two important "links" in this hypothetical chain.

Both the sex change *race* and the sex change window that opens up during the early stages of that race are relatively longer in seabream when compared to other species in which sex change occurs. It seems that in May and July, during the quiescent gonad stage, dominance hierarchies are correlated with the amount of brain GnRH-1 (**Chapter 4**). Wong (2006) showed that in June, the amount of gonadal aromatase expression is increased, which he suggested marks a crucial event that defines the sex change window. It is possible that dominant animals could undergo gonad development earlier (e.g. begin oogenesis faster), progressing towards gonad commitment and sex change before the submissive individuals. By June, when the gonadal aromatase begins to peak in the ambisexual gonad, sex in some animals might be decided.

Later, in July, increased expression of GnRH-3 is observed in the brain of ambisexual fish, followed by increased AVT expression in August (**Chapter 3**). How these events

might be related is unknown, but GnRH-3 seems to modulate olfactory and visual information (Zhang and Delay, 2007; Grens et al., 2005; Wielchmann and Wielchmann, 2001; Yamamoto and Kawashima, 1997). GnRH-3 may also have a role beyond affecting sensory pathways, such as modulating reproductive behavior *per se* (Ogawa et al., 2006). Perhaps, this rise in GnRH-3 levels, indicates that the animals are preparing to receive the visual and olfactory cues that regulate sex ratios.

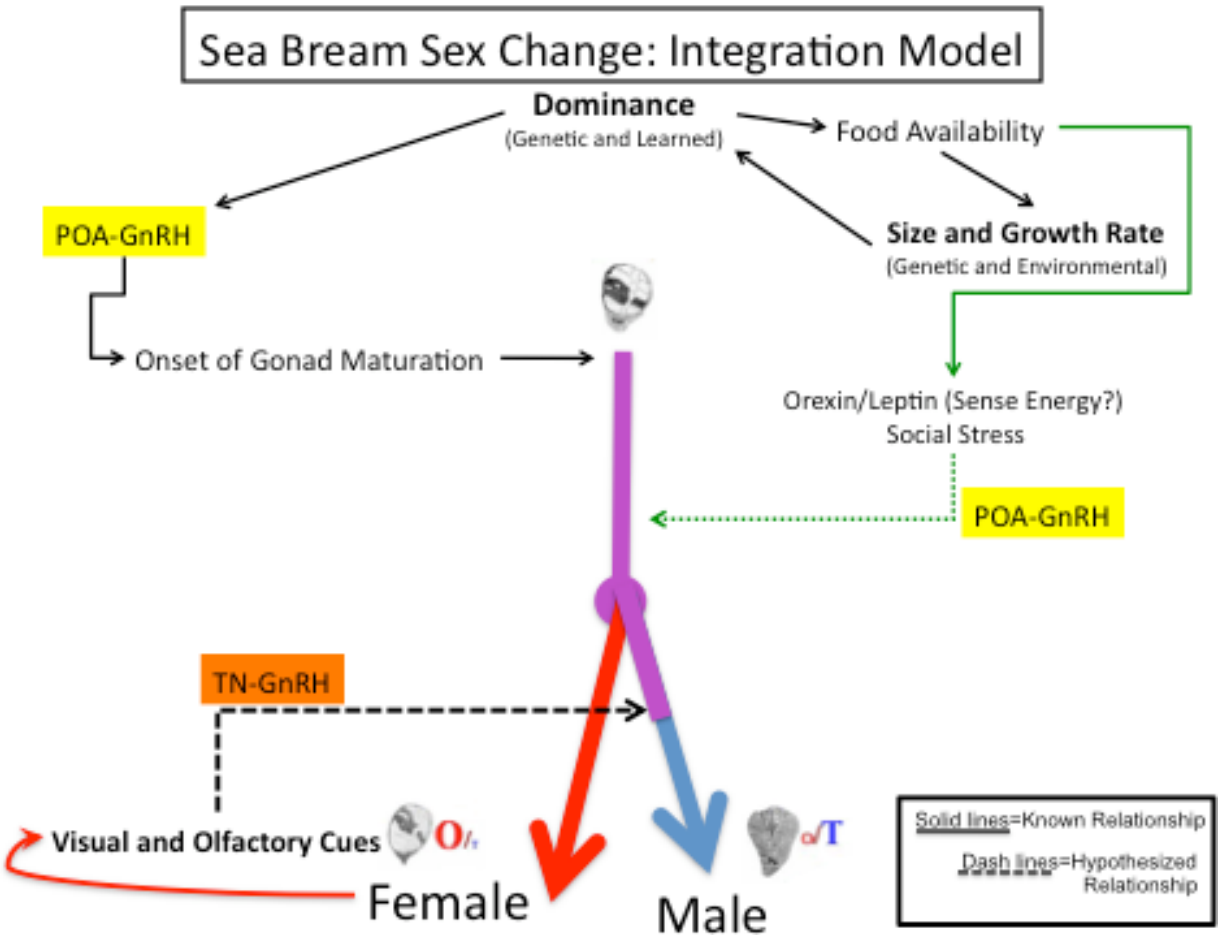


Figure 49. An alternative model that accounts for the possible role of GnRH-1 in seabream sex change and that considers the observed pattern of sex change seen in captive seabream, in which size is not a strong determinant of the outcome of sex change. Notice that GnRH-1 can serve by itself as an integrator of some of the putative parameters that might influence sex change.

I showed that color markings in the fish are sexually dimorphic, and that the pattern of expression also diverges between male and females (**Chapter 3**). In males, the

orange spot increases its intensity before spawning but disappears afterwards. The orange spot in the females does not disappear during spawning. The yellow stripe only seems to appear in females, and reaches peak intensity during spawning. Together, these observations suggest that during the final stages of sex change decision, months before spawning, the individuals might be able to use visual cues to assess the sex change status of those fish around them.

These results can be pieced together into a new model for sex change in seabream (**Figure 49**). In this model, size and age act as proximate factors to dominance. Dominance is influenced by size, which in turn depends on growth rate and energy reserves. Growth rate can depend on dominance, but is presumed to have genetic components.

In the first stages of sex change decision, dominance affects GnRH-1 content, which in turn affects the timing of sex change. Dominant fish begin to change sex first. Ambisexual fish have a higher impetus to begin gonad development earlier, because they have the potential to become male or females. Terminal females do not retain the capacity to change sex, so they have no reason to "rush" into gonad development. Thus, the observation that (smaller) ambisexual males are more dominant than (bigger) older regressed females, suggest that those males that succeed in showing dominance against females, may reach sex change faster. However, these dominant fish only increase their chance of becoming females.

In a later stage of sex change decision, the visual cues given out by advancing females, might force some fish to remain ambisexual. Those animals that reach gonadal committal will not be able to revert. Thus, in this model dominance becomes an

important factor but does not solely determine sex change. This model allows for growth rate, food availability, energy reserves, and other factors, to act on the HPG axis at different points along the way. The model is presented as an integrative model, in which multiple factors interact, each with its own window of action, to determine sex. The main axis affected by these factors is the HPG axis.

Future Directions

Although I successfully tested the 3 main hypotheses that I set out to explore in the beginning of this document, the main question that this thesis attempted to answer remains open: Can dominance behavior influence sex change? It is clear that dominance plays a role in seabream sex change, but its exact contribution is not known. This thesis laid the foundations for further exploration of this question.

To provide a more definitive model of sex change, and to ascertain the exact contribution of dominance to sex determination in seabream, it would be necessary to repeat the experiment described in **Chapter 4,** but with a slightly different approach. First, the number of groups should be increased and they should be kept in completely isolated systems (i.e. not share water). Second, more physical parameters should be measured including, weight of fish, fish color (orange spot and yellow stripe), and if possible abdominal ultrasound could be used to estimate the stage of gonadogenesis. An alternative approach could be to estimate GSI using the data set in the hormonal seasonal profile experiment (**Chapter 3**) to construct a regression model for it, based on estradiol and testosterone levels. Finally, the experiment should consist of: several groups composed of ambisexual fish only; at least one group composed entirely of regressed females; and two or more groups that have a mixed composition.

One large unknown is, how do the females "communicate" with the ambisexual fish in order to regulate sex ratios? This work revealed the existence of body markings that could act as cues, but the markings in the regressed females were not studied. Do they appear earlier than in younger, immature fish? Do they differ in intensity? The experiment that I just described could provide answer to these relevant questions.

I showed that GnRH-1 might be involved in sex change because dominance hierarchies correlate significantly with whole-brain GnRH-1 content. To test what the role is, a two-part experiment could be performed. Fish could be socially manipulated to change their "position" in the hierarchy to test if GnRH-1 *responds* to this change by being downregulated. If this is the case, the results of such manipulations could be tested on sex change outcome.

A similar role was observed here for GnRH-2. Specifically, it was shown that both GnRH-1 and GnRH-2 are correlated with rank, but each at different stages of the gonad cycle. The above described experiments of social manipulation could be repeated during spawning under two different conditions, one with intact gonads and another with gonadectomy. Furthermore, with a larger number of animals, the question of whether females or males respond differently to this behavioral influence could be addressed.

Finally, if sex change in the Gilthead seabream is a protracted event with multiple regulatory points, it would be useful to know where these regulatory points occur and what the contribution of each of these putative factors is to sex change. By manipulating some of the proposed variables in this model (e.g. manipulating the social environment, manipulating the fish with hormone implants that might increase coloration such as estradiol, 11-ketotestosterone or even testosterone, etc) at different times along the sex

change window, a better understanding of sex change in hermaphrodites would emerge. Alternatively, a non-linear statistical model could be built, based on the multiple variables described (size, growth rate, dominance rank, GSI, etc) which serve as putative proximate factors that reflect the ultimate cues that drive sex change, and the outcome of sex change observed in the above-described experiment. For example, an artificial neural network could be provided with the parameters and then trained using the outcome of sex change. If the network can successfully model sex change based on the input parameters, then a model for sex change with each factor having a quantifiable contribution could be built. Such a model could provide the *probability* of sex change based on the given parameters. The results of this modeling could be used in the aquaculture of seabream to achieve optimal sex ratios by manipulating the broodstock.

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