Reproductive success of a marine teleost was correlated with proactive and reactive stress coping styles

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Abstract

It has been observed that stress coping styles influence different biological aspects of fish, such as reproduction, highlighting a need to understand this relationship to improve the selection and reproductive success of breeders in aquaculture production. The present study investigated the relationship between reproductive success and stress coping styles in gilthead seabream (*Sparus aurata*) in captivity. To characterize stress coping styles, a total of 22 breeders were submitted to three different individual-based tests, one group-based test and post-handling glucocorticoid quantification. To assess spawning participation, a microsatellites analysis was performed on a total of 2698 larvae, which allowed each offspring to be assigned unambiguously to a single parental couple. Overall, gilthead seabream showed defined proactive and reactive behavioural traits. Proactive breeders exhibited higher levels of activity and risk taking and lower glucocorticoid blood levels than reactive breeders. The stress coping style traits were consistent over time and context (different tests). Breeders that contributed to a higher number of progeny exhibited proactive behaviours, while those showing low progeny contribution exhibited reactive behaviour. Therefore, breeders with a high proportion of progeny (>20%) had significantly higher activity and risk taking and lower cortisol than breeders with low progeny contribution (<20%). In addition, males were more proactive than females and males exhibited significantly higher activity, risk taking and lower cortisol than females. This study is the first to establish in gilthead seabream breeders: (a) a relationship between stress coping styles and spawning success, (b) a relationship between stress coping styles and gender, (c) the existence of proactive and reactive traits at the adult stage.

Keywords**:** behavioural traits, intra-individual consistency, reproduction, mate choice, physiology

Introduction

The reproductive strategies of fishes are diverse compared to other species, such as mammals or birds (Taborsky & Brockmann, 2010; Exbrayat, 2014). This variability stems from the fact that fish exhibit many different and often opposing tactics in aspects that, when grouped together, form the reproductive strategy of a species: sex differentiation (gonochoristic/hermaphroditic), size and age at first maturity, nutritional requirements, maturation in relation to environmental changes, spawning frequency (synchronous/asynchronous ovarian development), spawning behaviour (paired or group spawning), egg care/ egg size (*i.e.*, oviparity, viviparity, semelparity, iteroparity, etc.) (Murua & Saborido-Rey, 2003; Schuett et al., 2010; Duncan *et al.*, 2013). In accordance to Taborsky and Brockmann (2010) and Wolf *et al.* (2007), these reproductive strategies have evolved from natural selection pressures, life-history traits and adaptations to different environments. Reproduction is highly influenced by environment, physiology and nutritional status of individuals (Sumpter, 1997; Izquierdo *et al.*, 2001; Zohar & Mylonas, 2001; Mañanos *et al.*, 2008; Duncan *et al.*, 2013).

Behaviour is a biological factor that significantly influences reproduction and the genes that are passed to the following generation (Taborsky & Brockmann, 2010; Réale *et al.*, 2010; Harris *et al.*, 2010; Schuett *et al.*, 2010). In a broad sense, reproductive behaviour involves seeking and defending territories that offer advantages for spawning or offspring survival, mate selection and courtship, which ultimately increase reproductive success and offspring survival (Godin & Dugatkin, 1996; Wolf *et al.*, 2007; Harris *et al.*, 2010; Schuett *et al.*, 2010; Ariyomo & Watt, 2012). Thus, individual behavioural tactics, such as dominance, aggression, decision making and rank status, are employed by individuals to maximize their reproductive success (Biro & Stamps, 2008; Smith & Blumstein, 2008; Schreck, 2010; Schuett *et al.*, 2010; Smith & Blumstein, 2012;

Desjardins *et al.*, 2012). Stress coping styles are behavioural syndromes that are influenced by genetics and life-history experiences and which encompass a variety of behavioural tactics (Koolhaas *et al.*, 1999; Stamps, 2007; Øverli *et al.*, 2007; Schreck, 2010).

In broad definition, stress coping styles establish that individuals of a population vary in their behavioural responses to hazard situations and that these responses are consistent over time (Koolhaas *et al.*, 1999). Stress coping styles (SCS) vary from two extremes defined as proactive and reactive. Overall, proactive individuals are characterized to actively react to novel stimuli, are prone to take risk (Wilson *et al.*, 2010), show high levels of activity (Wilson & Godin, 2009; Castanheira *et al.*, 2013), tend to be offensive towards conspecifics (Koolhaas *et al.*, 1999; Huntingford *et al.*, 2010) and produce lower concentrations of post-stress glucocorticoids (Øverli *et al.*, 2007; Raoult *et al.*, 2012), while the opposite behaviour traits are seen in reactive individuals. The advantages of being proactive or reactive are controversial, however, different models have proposed that proactive individuals tend to grow faster, possess a faster metabolism and show high immune response (Réale *et al.*, 2010). In contrast, reactive individuals are sensitive to environmental stressors, show high plasticity and flexibility to novel environments and their life expectancy is higher (Castanheira *et al.*, 2015). Similarly, some studies have suggested that SCS are linked to reproductive success/characteristics and vary between sexes (Réale *et al.*, 2007; Schuett *et al.*, 2010; King *et al.*, 2013). For instance, Godin & Dugatkin (1996) and Ariyomo & Watt (2012) reported that proactiveness is related to a higher reproductive success in guppys (*Poecilia reticulata*) and zebrafish (*Danio rerio*). However, caution is needed as some studies have not found a link between reproductive success and stress coping styles, for example Wilson *et al.* (2010) and Sih &Watters (2005) did not find correlations between proactiveness and reproduction success in the Eastern mosquitofish, *Gambusia holbrooki* and water strider *Aquarius remiges,* respectively. In relation to sexes, King *et al*. (2013) suggested that stickleback (*Gasterosteus aculeatus*) males were more prone to explore novel environments than females and concluded that male's behaviours were associated with proactive SCS. In an adaptive framework, those average behavioural differences between males and females have been proposed to depend on natural selection processes or individual life history experiences (Koolhaas *et al*., 1999; Réale *et al*., 2007). Altogether, these conditions of selective pressure resulted in distinct behavioural tactics between sexes used by fish to increase or, at least, maintain their populations.

Therefore, the purpose of the present study was to investigate whether spawning participation and progeny contribution was related to stress coping styles or not in gilthead seabream (*Sparus aurata*) held in captivity. This fish species is a model organism in aquaculture research, which has great scientific importance and a high production in the European aquaculture industry. According to Castanheira *et al*. (2013), juveniles of this species present defined proactive and reactive coping styles, but to date, no studies have been conducted on gilthead seabream breeders. Additionally, Ibarra-Zatarain & Duncan (2015) described the courtship and spawning behaviour of this fish species and reported that seabream showed a preference to spawn in pairs, which indicates how reproductive hierarchies that dominate contributions to progeny are established and maintained. In this sense, the present study aimed to consider whether and how seabream reproductive success is linked with stress coping styles. Differences in behavioural tactics between sexes and the consistency of both proactive and reactive behaviours of seabream breeders across time were also investigated.

Material and methods

Ethic statement

All experimental procedures on fish that formed part of this study were carried out in strict accordance with the Spanish and European regulations on animal welfare (2010/63/UE and Federation of Laboratory Animal Science Associations, FELASA) and were approved by the Animal Ethics Committee of IRTA. All blood samples were performed after anaesthesia (Tricaine methanesulfonate (MS-222) at 100 ppm) to facilitate humane handling of the animals, and all efforts were made to minimize suffering of fish. Similarly, the method for tagging the fish complied with the ethic committee of IRTA and tags were implanted after anesthetizing fish (MS-222 at 100 ppm) to reduce suffering. The tests used in this study were similar to normal farm practices of capturing and moving fish and were considered to inflict minimal pain, suffering or distress.

Fish maintenance

Seabream breeders were held at IRTA facilities for at least 5 years under standardized conditions. Twenty-two mature gilthead seabream (*Sparus aurata*), 12 females and 10 males, with a weight of 2.59 ± 0.15 kg (mean ± S.E.) and a length of 49 ± 4 cm (mean ± S.E.) were used for this study. Fish were tagged for identification with Passive Integrate Transporter tags (11.5 mm length x 2mm diameter (0.1g); PIT-ID-100B Microchip; TROVAN-ZEUS; Madrid, Spain). PIT tags were injected into muscle through a IM-200 syringe implanter (Trovan). Afterwards, fish were divided equally (by number and gender) among two 16.2 m^3 rectangular (6 × 3 × 0.9 m) fiberglass tanks (T1 and T2). Tanks were located outside in a greenhouse structure covered with shade netting. Tanks were totally covered and photoperiod was adjusted to follow the natural seasonal cycle by using two halogen white lights located inside of each tank. Lights turned on/off in tanks with a photocell sensor. During the experimental period water temperature and dissolved oxygen were maintained between 18-19°C and 5-6 mg/L, respectively. Fish were fed *ad-libitum*, daily in the mornings (between 0900:1000 hours), with a commercial balanced diet (Vitalis CAL-9, Skretting, Burgos, Spain).

Paternity analysis of larvae

Genetic fingerprinting was performed to identify spawning participation of breeders. A panel of 11 microsatellite markers (STRs) developed and validated on a group of known families analysed in previous work (Franch *et al*., 2006; Parati *et al*., 2010; Chavanne *et al*., 2012) was used in this study to analyse a total of 2698 larvae, approximately 200 larvae per spawn. Parentage assignments were established by the specific allocation software wHDP, which uses the exclusions and the likelihood-based approach (Galli *et al*., 2011; Chavanne *et al*., 2012). The spawns were analysed as follows: one spawn per week was selected from each week of the entire spawning season from January to April and six spawns from consecutive days in the middle of the spawning season. This sampling schedule was selected to give information on (a) the participation of breeders over the entire spawning season and (b) the participation at the peak (middle) of the spawning season when the most eggs were collected. Breeder participation above 20% parentage was considered as high and below 20% as low, this classification was confirmed after evaluating the progeny contribution data of breeders with a Discriminant Analysis, with Box´s (Box´s test; $F_{1,936} = 18.08$, $P < 0.001$) and Lambda Wilks/X^2 tests (Wilk´s/ Chi-square tests; $\lambda = 0.373$, $X^2_1 = 19.23$, $P < 0.001$).

Stress coping style evaluation

To characterize stress coping styles, three types of evaluation were used. In the first evaluation, individual fish were submitted to a series of three tests for profiling each individual's behaviour in a stressful situation. These three tests will be referred to as 'individual tests'. At the end of the three individual tests, the second type of evaluation was to determine stress hormone levels in blood samples taken from the fish. The third evaluation was made on a group of fish and will be referred to as a 'group test'.

Stress coping style individual tests

To characterize stress coping styles, fish were randomly selected and submitted to a series of three individual stress tests (Figure 1) applied one after another (to mimic the normal handling farming procedures). The first test, *restraining test*, consisted of netting fish individually inside the water and three behavioural variables were evaluated for 120 seconds: **i)** the first activity time (s), or latency time to move, of the fish once in the net (NetFirstAct), **ii)** the total activity time (s) of the fish spent moving or trying to escape from the net (NetTotAct) and **iii)** the total number of escape attempts (NetEsc) (*Solea senegalensis* Martins *et al*., 2011; *Sparus aurata* Castanheira *et al*., 2013; *Gasterosteus aculeatus* King *et al*., 2013; *S. senegalensis* Ibarra-Zatarain *et al*., 2016). The second test, *confinement test*, consisted of evaluating for 300 seconds the reaction of fish to a confinement situation (the fish was placed in a small tank (56 cm length x 36 cm width x 30 cm depth) with a constant flow through of oxygenated water) by evaluating two variables: **i)** the latency to the first activity time (s) of fish in confinement (ConLat) and **ii)** the total activity time (s) in confinement (ConAct) (*Lepomis macrochirus* Wilson & Godin, 2009; *Cyprinus carpio* Huntingford *et al*., 2010; *G. aculeatus* King *et al*., 2013; *S. aurata* Herrera *et al*., 2014; *Dicentrarchus labrax* Ferrari *et al*., 2015; *S. senegalensis*

Ibarra-Zatarain *et al*., 2016). The third trial, *anaesthesia test*, consisted of evaluating the time required (s) to induce 5 different anaesthesia levels (Table 1) (*Ictalurus punctatus* Welker *et al*., 2007; *Danio rerio* Nordgreen *et al*., 2014). The anaesthesia agent used was buffered tricaine methanesulfonate (MS-222) at a concentration of 100 ppm (Norambuena *et al*., 2011). To investigate the consistency of behavioural personalities (Castanheira *et al*., 2013; King *et al*., 2013; Ferrari *et al*., 2015), the three individual coping style assays were performed twice, in June and September, on all individuals.

Stress hormone evaluation

To evaluate stress hormone levels, a blood sample (0.5 ml) was extracted from the caudal vein of anesthetized fish after completing the individual tests in the first trial (June) to quantify cortisol, lactate and glucose concentrations (Figure 1). To avoid blood coagulation and protein degradation, a solution of 10 µl sodium heparin (5%, 25.000 UI; HOSPIRA) and 15 µl aprotinin (from bovine lung; 0.9% NaCl, 0.9% benzyl alcohol and 1.7 mg of protein; SIGMA) was placed inside eppendorf tubes, while the syringes and needles were coated with heparin. Blood samples were centrifuged (ThermoScientific centrifuge, M23i; Thermo rotor AM 2.18; 24 x 1.5 ml) at 3000 g and 4º C for 15 min and the plasma supernatant was removed and stored in triplicate at −80º C prior to analysis (Martins *et al*., 2011; Ibarra-Zatarain *et al*., 2016). Cortisol was measured by a competitive conjugated binding ligand by means of a commercial ELISA kit (Range of detection: 0-800 ng/mL; DEMEDITEC, Kiel-Wellsee, Germany), whereas glucose and lactate were measured by means of commercial enzymatic colorimetric kits (SPINREACT, Gerona, Spain). Cortisol, glucose and lactate absorptions were read by a spectrophotometer (Infinite M-200; TECAN, Switzerland), at 23ºC and 505 nm.

Stress coping style group test

One month after completing individual tests of trial 1 (in June), a single risk-taking group test was performed (Figure 1). This test aimed to determine fish capacity to take risk by crossing from a known area (safe zone) to an unknown area (risky zone) during a 24 h period (fish were acclimated previously for 8 hours). The test was realized in a 16 m^3 tank (6 m length x 3 m width x 0.9 m depth), divided into two equal areas by a wooden screen. A window (30 cm width x 30 cm tall) was placed at the middle part of the dividing wood barrier with a door that could be opened to allow fish to pass from an area to another. The window was at the centre of a passive integrated transducer tag reading antenna (SQR series; TROVAN-ZEUS, Madrid, Spain) that was positioned to read the tag number of the fish that passed through the window to the risk zone. In addition, the safe zone was isolated from light (3 lux on the surface) to provide a comfortable and secure space for fish. In contrast, the risky area was illuminated (15 lux OSRAM DULUX 150W). The test was repeated separately for each group of seabream. Breeders that successfully crossed were defined as proactive, while fish that did not cross were labelled as reactive. To reduce the possibility of interference of transient motivational states with individual behavioural responses, fish were fed *ad libitum* in the evening before testing, therefore, all individuals were, presumably, equally motivated for feeding. This test, which has been widely used to discriminate personalities, and definitions of behaviours, were adapted from other studies performed with seabream (Castanheira *et al*., 2013) and other fish species such as, bluegill sunfish (Wilson & Godin, 2009), mulloway *Argyrosomus japonicus* (Raoult *et al*., 2012), European seabass (Ferrari *et al*., 2015) and Senegalese sole (Ibarra-Zatarain *et al*., 2016).

Statistics

Data from the three individual tests from the two experimental groups trials were submitted to three successive principal component analyses (one per test) and reduced into three principal component scores (PCS1, PCS2 and PCS3) corresponding to restraining, confinement and anaesthesia tests, respectively. Each calculated principal component score (PCS 1, 2, 3) gathered the variables and represented fish individual behaviour in each test. Then, a general linear multivariate model (GLMM) was performed on the data to identify differences between PCS 1, 2, 3, blood parameters and risk taking (a categorized value identifying fish that crossed or did not cross in the group test), to spawning participation and sex. The intra-individual repeatability, between trials 1 and 2 (June and September), was assessed for the three individual tests by performing a repeated measures analysis with a general linear model (RM-GLM). A student's *t*-test was performed to assess possible behavioural differences between males and females. Lastly, a Pearson analysis was performed to evaluate correlations between PCS 1 to 3 and cortisol, glucose and lactate concentrations and to determine correlations of individual tests between both trials. Results were expressed as mean ± standard error of the mean (S.E.M) and significant differences were determined with $P<0.05$. Statistics were performed with SPSS 18 (IBM).

Results

Paternity assignment

The reconstruction of sibship or relationship between siblings and parents permitted us to assign unambiguously each offspring to a single parental couple, with a 99.9 % of assignments solved in a single match by exclusion approach and only a 0.1% of multiple match assignment solved by stochastic approach. A total of 10 females and 9 males reproduced successfully: eight fish contributed more than 20%, 11 contributed less than 20% and only three individuals did not spawn.

Coping style characterization

Gilthead seabream breeders showed consistent individual differences in behaviour that resembled the extremes of coping styles (proactive vs reactive), since some individuals were highly active and others remained immobile during the individual tests (Table 2). The ten breeders that crossed from the safe to the risky area (5 males / 5 females) in the risk-taking test showed significantly higher scores for restraining PCS1 ($F_{1, 14} = 5.66$, $P = 0.022$) and confinement PCS2 ($F_{1, 14} = 5.66$, $P = 0.022$) tests and had lower cortisol ($F_{1, 14} = 8.75$, $P = 0.019$) and glucose ($F_{1, 14} = 25.25$, $P = 0.001$) levels than the twelve fish that did not cross (Figure 2). Gilthead seabream showed high intra-individual repeatability in NetFirstAct (Wilk´s test; $\lambda = 0.868$, $F_{1, 21} = 0.700$, $P = 0.432$), NetTotAct (Wilk´s test; $\lambda = 0.992$, $F_{1, 21} = 0.161$, $P = 0.693$), NetEsc (Wilk´s test; $\lambda = 0.979$, $F_{1, 21} = 0.443$, $P = 0.443$), ConLat (Wilk´s test; $\lambda = 0.757$, $F_{1, 21} = 0.728$, $P = 0.594$), ConAct (Wilk´s test; $\lambda = 0.972$, $F_{1, 21} = 0.609$, $P = 0.444$) and ANE3a (Wilk´s test; $\lambda = 0.959$, $F_{1, 21} = 0.038$, $P = 0.847$) between trials 1 and 2 (Table 2). Significant and high correlations were found between trial 1 (June) and 2 (September) in restraining (NetFirstAct $R^2 = 0.914$, $P = 0.001$, NetTotAct $R^2 = 0.746$, $P = 0.001$ and NetEsc $R^2 = 0.413$, $P = 0.048$), confinement (ConLat $R^2 = 0.685$, $P = 0.001$ and ConAct $R^2 = 0.898$, $P = 0.001$) and anaesthesia (ANE3a $R^2 = 0.541$, $P = 0.009$) parameters (Figure 3). Further, the principal component scores of restraining and confinement tests (PCS1 and PCS2) were significantly correlated ($R^2 = 0.423$, $P = 0.037$), demonstrating the existence of behavioural syndromes consistent across contexts in this fish species. Altogether, these results confirmed the existence of proactive and reactive stress coping styles behaviours in gilthead seabream.

Coping styles and spawning participation

The eight seabream presenting high spawning participation showed significantly higher scores for restraining PCS1 ($F_{1,\ 14}$ = 7.91, P = 0.020) and confinement PCS2 ($F_{1,\ 14}$ = 22.18, P = 0.001) and lower cortisol ($F_{1,\ 14}$ = 4.81, P = 0.037) and lactate ($F_{1,\ 14}$ = 5.63, P = 0.033) levels than the 14 fish presenting low spawning participation (Table 3, Figure 4). In the risk-taking test, 100% fish that crossed (n = 10) successfully spawned, against 75% for fish that did not cross (n = 12). The proportion of fish that contributed to more than 20% to progeny generation represented 70% of those fish that crossed, whereas only 8% of fish that did not cross. Therefore, gilthead seabream with high progeny contribution were shown to exhibit behavioural traits that corresponded to proactive SCS traits, while behavioural styles of breeders with low spawning participation resembled reactive stress coping styles.

Coping styles and sex

Overall, seabream males performed significantly more escape attempts in the restraining test and were more active in the confinement test than the 12 females. Males presented significantly higher scores for restraining PCS ($F_{1,\ 14}$ = 13.60, P = 0.002) and confinement PCS ($F_{1,\ 14}$ = 6.82, P = 0.027) tests and had significantly lower cortisol levels (102.71 ± 10.82 ng/ml) than females (142 ± 12.25 ng/ml) ($F_{1,\ 14}$ = 9.45, P = 0.008). These behavioural characteristics suggest that males were more prone to proactive SCS and females to reactive strategies (Figure 5). No significant correlations were observed between SCS and size (weight or length) of fish.

When evaluating males and females by their progeny contribution, males with high contribution (*n* = 3) presented significantly higher scores in restraining PCS (two sample t-test; *t* = 6.288, *P* = 0.012, df = 2), confinement PCS (two sample t-test; *t* = 3.210, *P* = 0.042, df = 2) and anaesthesia PCS (two sample t-test; *t* = 6.599, *P* = 0.011, df = 2) and lower cortisol (two sample t-test; *t* = 5.896, *P* = 0.028, df = 2) and lactate levels (two sample t-test; *t* = 4.870, *P* = 0.040, df = 2) than males with low progeny contribution (*n* = 7). Similarly, females with high fingerling contribution (*n* = 5) differed from females with low contribution (*n* = 7) in confinement PCS (two sample t-test; *t* = 3.920, *P* = 0.017, df = 4), anaesthesia PCS (two sample t-test; *t* = 3.525, *P* = 0.009, df = 4), cortisol (two sample t-test; *t* = 6.611, *P* = 0.003, df = 4), glucose (two sample t-test; *t* = 8.465, *P* = 0.001, df = 4) and lactate (two sample t-test; *t* = 3.525, *P* = 0.009, df = 4), respectively (Figure 6). Therefore, it was observed that males and females with high contribution tended to exhibit proactive SCS, while the opposite was observed for those that contributed with less progeny.

Discussion

Coping styles characterization

Captive gilthead seabream breeders presented two main consistent behaviours in response to the different coping style tests performed. The individual tests identified fish with different activity levels and distinct physiological changes, which represented behavioural styles ranging from proactive and reactive traits (Koolhaas *et al.*, 1999; Øverli *et al.*, 2007; Castanheira *et al.*, 2015; Ibarra-Zatarain *et al.*, 2016). Similarly, the risk-taking test determined individual behavioural differences between coping styles of fish that crossed, defined as proactive, and those that did not cross categorized as reactive (Harris *et al.*, 2010; Réale *et al.*, 2010). Importantly the individual and group coping style tests categorized the same individuals as proactive and reactive showing consistency over different tests or context. The behavioural characteristics of proactive and reactive gilthead seabream breeders were in line with those previously reported in the same fish species (Castanheira *et al*., 2013) and in other species at different life stages, such as bluegill sunfish (Wilson *et al*., 2009), common carp (Huntingford *et al*., 2010), European seabass (Ferrari *et al*., 2015), rainbow trout (Wilson & Stevens, 2005) and zebrafish (Tudorache *et al*., 2013). The individual behavioural differences in gilthead seabream breeders appear to represent non-adaptive variations and might be the result of differences in neuroendocrine profiles, fitness, curiosity, dominance, motivation or genetic, as it has been hypothesized in previous studies related to SCS (Koolhaas *et al*., 1999;, Øverli *et al*., 2007; Réale *et al*., 2007; Herrera *et al*., 2014), although this assumption must be further examined in this fish species at this life stage. Additionally, gilthead seabream showed significant cross-context correlations between PCS 1 (restraining test) and 2 (confinement test). The gilthead seabream also exhibited intra-individual behavioural repeatability and consistency over time with correlations in the restraining, confinement and anaesthesia tests between trials 1 (June) and 2 (September). These results are in line with those reported in the same species (Castanheira *et al*., 2013) and in other fish species such as the bluegill sunfish, European seabass and Senegalese sole (Wilson & Godin, 2009; Ferrari *et al*., 2015; Ibarra-Zatarain *et al*., 2016). Thus, the behavioural consistency over time and across contexts observed in the present work corresponds to the definition of stress coping styles (Koolhaas *et al*., 1999; Wilson & Godin, 2010; Ferrari *et al*., 2015) and reinforces their existence in this fish species at adult age. Nonetheless, further studies are recommended to evaluate if stress coping styles remain consistent over time in seabream breeders that change sex, which is a characteristic of this fish species.

Coping styles and reproduction

Some theories suggest that behavioural differences between fish presenting distinct spawning successes are expected, since individuals are influenced by social status, environmental conditions and use diverse mating strategies (Biro & Stamps, 2008; Smith & Blumstein, 2008; Schuett *et al*., 2010; Conrad *et al*., 2011). In the present study, the genetic pedigree reconstruction has permitted an unambiguous and thorough investigation of the family structure in a mass spawning batch, allowing detection of 19 gilthead seabream that successfully spawned and only three individuals which did not contribute to the analysed progeny. Moreover, when breeders were evaluated by their progeny contribution (high and low), fish demonstrated different coping abilities to stressful situations. Fish with high progeny contribution exhibited behaviours that were characterised by proactive SCS, whereas fish with low offspring contribution exhibited significantly less activity, higher cortisol, etc., behaviours that characterise reactive SCS. Therefore, in the present study under a captive environment, fish (male and female) that exhibited behaviours that characterise proactive SCS had also higher reproductive success, which may be related to proactiveness being an indicator of high fitness, energy, dominance and disease resistance (Biro & Stamps, 2008; Sih & Bell, 2008; Réale et al., 2010; Schuett et al., 2010; Martins et al., 2011), which would benefit reproductive success in a stable culture environment. Similar observations to those described in the present study have been reported in other fish species, such as guppies *Poecilia reticulata* (Godin & Dugatkin, 1996), three spined stickleback (Candolin, 1999) and African cichlids *Astatotilapia burtoni* (Maruska & Fernald, 2013). These authors reported that proactive males were preferentially selected by females and, therefore, had higher reproductive success. Nevertheless, the role of reactive fish in reproduction should not be neglected, since microsatellites analysis revealed that reactive breeders did also spawn, although their contribution to progeny was not as high as proactive fish. A possible hypothesis for explaining why reactive breeders, both males and females, contributed fewer progeny might rely on their reproductive tactics, such as sneaking and cooperation. Sneaking and cooperation provide higher opportunities to fish to become reproductively competent in aversive situations and have been observed in different taxa (butterflies *Pierisnapi*, Wiklund *et al*., 2001; hyaenas, *Crocuta crocuta*, East *et al*., 2003; coho salmon, *Oncorhynchus kisutch*, Watters, 2005; bluegill, *Lepomis macrochirus*, Charnov, 2009). According to Taborsky *et al*. (2008) and Taborsky & Brockmann (2010), the evolution of sneaking and cooperation may be subject to intra-sexual competition (*i.e.*, dominance vs. subordination) and may have evolved from similarly long-term to permanent behavioural, morphological, physiological and environmental adaptations. In the case of gilthead seabream, Ibarra-Zatarain & Duncan (2015) observed a certain degree of dominance during the courtship and spawning rush. Therefore, when considering possible hierarchies developed by proactive gilthead seabream during spawning, reactive breeders were thought to implement these alternative reproductive tactics to increase their reproductive opportunities, to reduce their energetic cost and physical risk (*i.e.*, injuries). Another consideration is that the culture environment is a safe environment free from predation and with good food availability, which would favour a proactive strategy. A different environment with high risk and low food availability (such as a natural environment) may favour reactive strategies. Reactive strategies have been shown to have higher survival (Castanheira *et al*., 2015) and may, therefore, in a high-risk environment result in more reactive breeders, compared to proactive breeders, attaining maturity and spawning. Previous and present results suggest that the balance of both SCS strategies into a population is fundamental within several fish species, since they represent **i)** different adaptive advantages and solutions to complex situations or environments and **ii)** different social relationships and cooperation between individuals (Wiklund *et al*., 2001; Réale *et al*., 2007; Taborsky *et al*., 2008; Schuett *et al*., 2010; King *et al*., 2013; Muraco *et al*., 2014).

Coping styles and sex

Male breeders (*n* = 10), exhibited behavioural tactics that were consistent with proactive traits, while females behaviours (*n* = 12) were similar to reactive SCS, by considering aspects of overall activity, exploration, risk taking disposition and glucocorticoids concentrations. Similar observations of a more proactive behaviour in males than in females have been reported in guppies (Harris *et al*., 2010) and three spined-sticklebacks (King *et al*., 2013). A possible explanation for these behavioural differences between sexes relies on distinct life history strategies and adaptations of males and females. Several hypotheses have specified that males possess a higher physical fitness, are more aggressive and dominant-oriented towards conspecifics, take higher risk to get mating partners and generally invest lower energy and time in offspring than females (Candolin, 1999; Schuett et al., 2010; King et al., 2013). Therefore, males with low investment in each gamete aim to compete and spawn with as many females as possible and proactive strategies would help this aim. By contrast, females make a high investment in each gamete, have fewer total number of gametes and cannot spawn with many partners, which results in females aiming to spawn with the most genetically fit male available. Therefore, females are frequently surrounded by potential and competing mating partners, leading females to forage less and save more energy to maximize their physical fitness, improve their reproductive success and produce a higher number of offspring throughout their life (Smith & Blumstein, 2008; Schuett *et al*., 2010; King *et al*., 2013). In the present study, these distinct adaptive features were suggested to be reflected by differences in behaviours and reproductive characteristics between sexes (Godin & Dugatkin, 1996; Biro & Stamps, 2008; Sih & Bell, 2008; Smith & Blumstein, 2008, 2012; Schuett *et al.*, 2010; Ariyomo & Watt, 2012). Interestingly, when seabream was analysed by their progeny contribution, not individually, it was observed that males and females with high progeny contribution presented behaviours that resembled proactive stress coping styles (higher activity, risk taking, less cortisol production), while the opposite was observed for males and females with low progeny production. A possible explanation of this pattern could rely on the fact that when males court and mate with females with similar behavioural personalities, the reproductive success, fecundity and number of offspring produced is regularly higher than in disassortative couples (Schuett *et al.*, 2010; Ariyomo & Watt, 2012). A probable hypothesis for this reproductive advantage is based on the fact that coordinated and similar behaviours may result in higher physical fitness (*i.e.*, energy, metabolism, etc.) and may reduce the energetic cost of reproduction (*i.e.*, conflicts, injuries, mate competition, detrimental consequences of social stress, etc.).

Males and females with high offspring contribution had significantly lower glucocorticoid levels than breeders with low progeny generation. High and low cortisol levels are also associated with reactive and proactive SCS, respectively, and reflect the ability of organisms to cope or approach with stressful situation (Øverli *et al.*, 2007; Raoult *et al.*, 2012). Additionally, different studies have demonstrated a relationship between reproduction and cortisol concentration. For instance, Norris & Hobbs (2006) stated that variations in glucocorticoid concentrations that resulted from detrimental situations and chronic stress situations, affected fish gonadal development and reproduction. Similarly, Cook *et al.* (2011) observed that pink salmon (*Oncorhynchus gorbuscha*) that successfully spawned had lower cortisol levels than unsuccessful individuals, being in line with the observations made in the present study. Lastly, Foo &

Lam (1993) and Pottinger *et al*. (1995) reported that reproductive hormones, such as serum testosterone in tilapia (*Oreochromis mossambicus*) and 17ß-estradiol in rainbow trout (*Oncorhynchus mykiss*) were inhibited by cortisol once exposed to a stressful situation and this alteration affected the reproductive success of both fish species. These findings led to the hypothesis that cortisol production might be more efficiently negatively regulated in proactive seabream breeders faced with stressful situations than in reactive animals, preventing the inhibition of reproductive hormones and resulting in higher dominance, spawning participation and reproductive success. In contrast, reactive fish might use more energy to maintain a basal cortisol homeostasis in stressful situations than proactive individuals to spawn and reproduce. However, this previous assumption should be further investigated in gilthead seabream, in particular, it would be interesting to implement the present information with studies evaluating the correlation between glucocorticoid levels and key reproductive hormone levels, in gilthead seabream breeders, before and after stressful situations.

The present study demonstrated for the first time that seabream breeders presented defined proactive and reactive behaviours and a high behavioural consistency over time and across contexts. However, the most remarkable result of the present study was the significant relationship between SCS and spawning participation, indicating that proactive gilthead seabream participated more actively in the generation of progeny than reactive fish. In addition, males and females showed different behavioural patterns, since males presented higher activity, reacted faster to novel situations and produced lower glucocorticoid levels than females. Moreover, males and females with high and low progeny contribution exhibited different coping abilities to stressful situations that resulted from defined proactive and reactive strategies. Lastly, further field experiments are recommended to evaluate if SCS remains consistent in seabream breeders that change sex.

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Author contribution

Conceptualization: Z.I.Z., N.D., K.P., S.C.; Methodology: Z.I.Z., N.D., K.P., S.C.; Formal analysis: Z.I.Z., N.D., K.P., S.C.; Investigation: Z.I.Z., N.D., K.P., S.C.; Data curation: Z.I.Z., N.D., K.P., S.C.; Writing - original draft: Z.I.Z., N.D.; Writing - review & editing: Z.I.Z., N.D., K.P., S.C.; Visualization: Z.I.Z., N.D.; Supervision: N.D.; Project administration: N.D., KP; Funding acquisition: N.D., K.P.

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Table 1. Description of the different anesthesia levels in fish and associated behaviors (Schoettger and Julin, 1967)

Anaesthesia levels	Abbreviation	Characteristic
1	ANE1	Partial loss of reaction to external stimuli
2	ANE2	Partial loss of equilibrium without reaction to stimulus
3a	ANE3a	Fish usually turn-over but maintain swimming ability
3b	ANE3b	Swimming activity stops but react to pressure in extremities
4	ANE4	Loss of reflex, no reaction to strong external stimuli

Table 2. General coping style of breeders in trial 1 and 2. Min, max and coefficient of variation (CV) were calculated from both trials. CV was calculated to represent the inter-individual variation (CV = standard deviation / mean * 100). NetFirstAct = Net first activity, NetTotAct = Net total activity, NetEsc = Net escapes, ConLat = Latency to move in confinement, ConAct = Total activity in confinement. na = not applied. Bold values represent high degree of consistency over time.

Tests	**Variables**	**Trial 1 (June)**	**Trial 2 (September)**	**Min**	**Max**	**CV (%)**	**Repeated ANOVA**			
							Wilk´s λ	**F**	**Df**	**P**
Restraining	NetFirstAct	51.4 ± 10.6	43.5 ± 9.1	1	120	20.7	**0.868**	**0.700**	**21**	**0.432**
	NetTotAct	16.3 ± 2.1	16.3 ± 2.3	1	52	13.5	**0.992**	**0.161**	**21**	**0.693**
	NetEsc	14.5 ± 2.2	16.8 ± 2.5	1	46	15.1	**0.979**	**0.443**	**21**	**0.443**
Confinement	ConLat	41.8 ± 10.8	35.3 ± 11.6	1	219	29.0	**0.757**	**0.728**	**21**	**0.594**
	ConAct	131 ± 34.4	123.2 ± 31.1	4	667	25.7	**0.972**	**0.609**	**21**	**0.444**
Anaesthesia	ANE 1	28.9 ± 1.51	31.6 ± 2.3	18	59	6.2	0.609	1.302	21	0.011
	ANE 2	59.2 ± 3.2	52.7 ± 3.1	31	91	5.6	0.725	0.987	21	0.037
	ANE 3a	108.6 ± 7.8	104.3 ± 5.1	50	189	11.8	**0.959**	**0.038**	**21**	**0.847**
	ANE 3b	182.3 ± 16.5	126.3 ± 6.7	81	368	7.5	0.968	12.98	21	0.002
	ANE 4	211 ± 16.1	166.5 ± 12.0	102	379	7.4	0.874	14.01	21	0.001
Blood analysis	Cortisol (ng/mL)	129.8 ± 11.9	na	47	249	9.3	na	na	Na	na
	Glucose (mmol/L)	8.01 ± 0.4	na	3	12	5.0	na	na	Na	na
	Lactate (mmol/L)	6.9 ± 0.5	na	3	12	7.2	na	na	Na	na

Table 3. Coping style responses averaged from trial 1 and 2 of breeders with high and low spawning participation. NetFirstAct= Net first activity, NetTotAct= Net total activity, NetEsc= Net escapes, ConLat= Latency to move in confinement, ConAct= Total activity in confinement. Letters indicates a statistical difference

Tests	**Variables**	**High spawning (*n* = 8)**	**Low spawning (*n* = 14)**
Restraining	NetFirstAct	30.1 ± 13.4	57.4 ± 13
	NetTotAct	20.3 ± 4.4^A	14.1 ± 2.2^B
	NetEsc	18.3 ± 4.1^A	14.2 ± 3^B
Confinement	ConLat	11.7 ± 3.1^A	53.9 ± 16.3^B
	ConAct	223.5 ± 72^A	72.1 ± 21.4^B
Anaesthesia	ANE 1	29.4 ± 3.5	30.7 ± 2.3
	ANE 2	54.6 ± 5.4	56.7 ± 4
	ANE 3a	106.4 ± 12	93.1 ± 7.5
	ANE 3b	179.5 ± 23.5	140.0 ± 11.3
	ANE 4	223.5 ± 29.8^A	168.8 ± 11.5^B
Blood analysis	Cortisol (ng/mL)	109.5 ± 13.7^A	135.7 ± 12^B
	Glucose (mmol/L)	7.6 ± 0.8	8.1 ± 0.5
	Lactate (mmol/L)	5.7 ± 0.5^A	7.5 ± 0.6^B

Figure caption

Figure 1. Chronogram figure explaining the different behavioural tests (restraining, confinement and anaesthesia) and group (risk taking) applied to gilthead seabream to characterize stress coping styles

Figure 2. Principal component scores and physiological differences of seabream that crossed and those that did not cross in the risk test. * Indicates significant differences

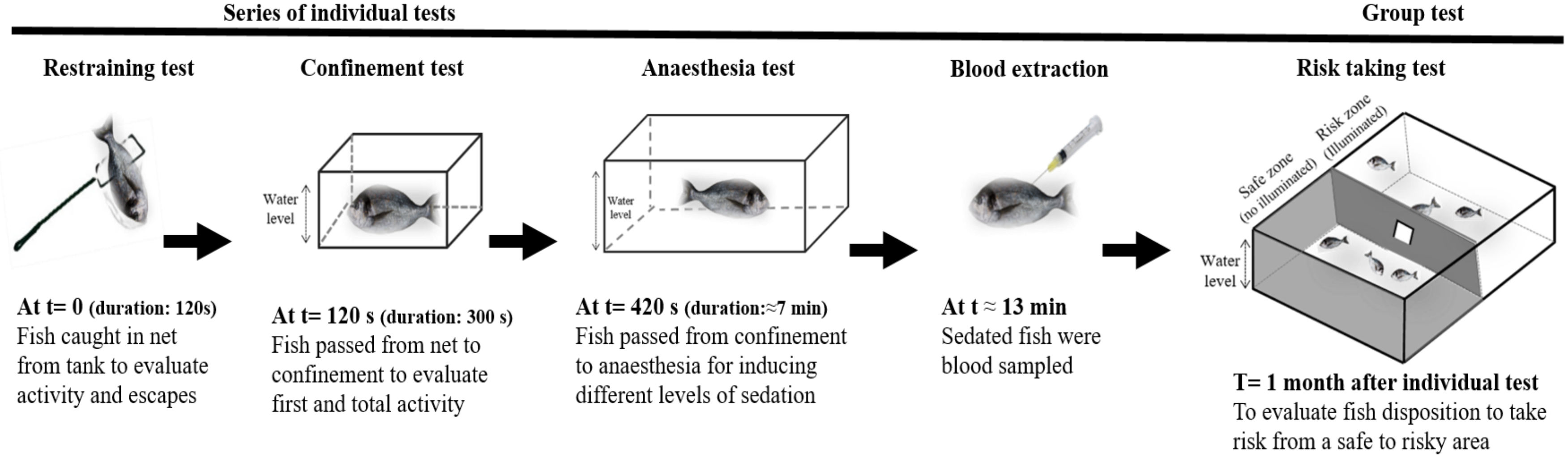
Figure 3. Significant correlations of gilthead seabream behavioral parameters between trials 1 (June) and 2 (September). A = Net first activity, B = Net total activity, C = Net escapes, D = Latency in confinement, E = Total activity in confinement, and F = Anaesthesia3.

Figure 4. General behavioural differences between behaviour of breeders with high (>20%; n = 8) and low (< 20%; n = 14) spawning participation by comparing their PCS. * Indicates significant differences.

Figure 5. Principal component scores and physiological differences in females and males of gilthead seabream breeders. * Indicates significant differences

Figure 6. Behavioural differences and glucocorticoids production of males (upper graph) and females (lower graph). Dark blue represents males and females with high progeny and light blue represent males and females with low progeny contribution. * Indicates significant differences

Figure 1



Series of individual tests

Group test

Restraining test

At t= 0 (duration: 120s)
Fish caught in net from tank to evaluate activity and escapes

Confinement test

At t= 120 s (duration: 300 s)
Fish passed from net to confinement to evaluate first and total activity

Anaesthesia test

At t= 420 s (duration:≈7 min)
Fish passed from confinement to anaesthesia for inducing different levels of sedation

Blood extraction

At t ≈ 13 min
Sedated fish were blood sampled

Risk taking test

T= 1 month after individual test
To evaluate fish disposition to take risk from a safe to risky area

Figure 2

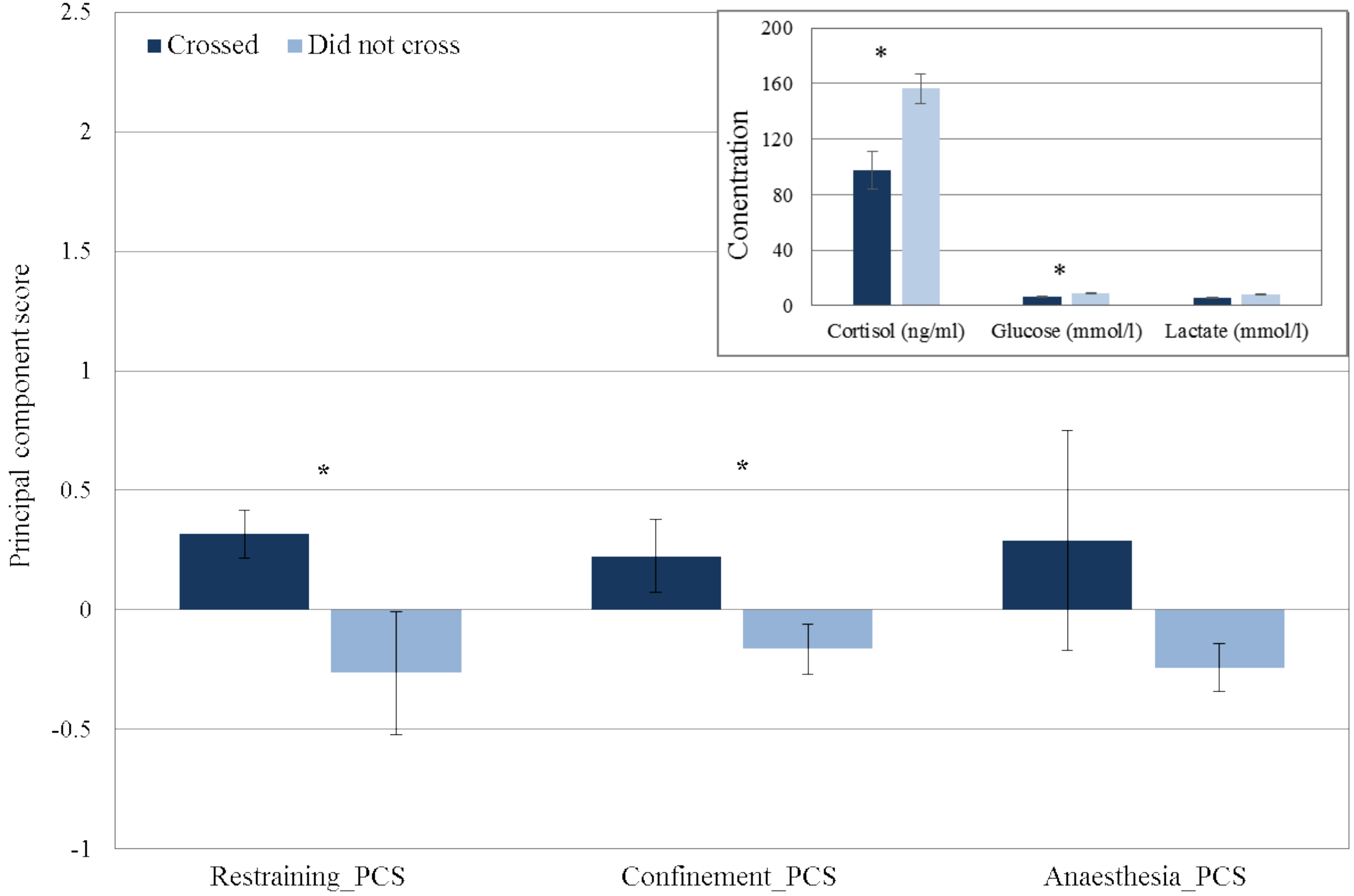
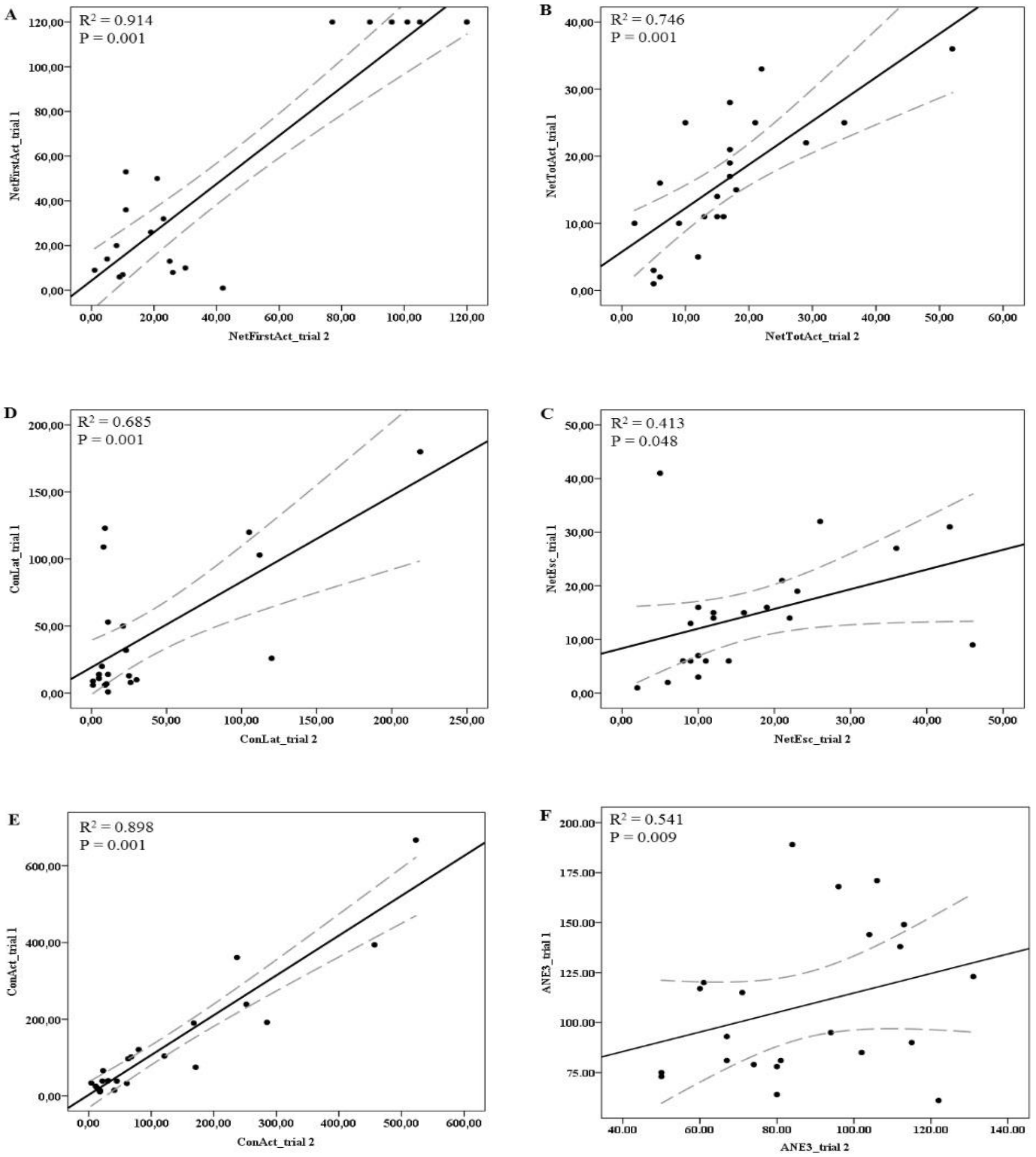
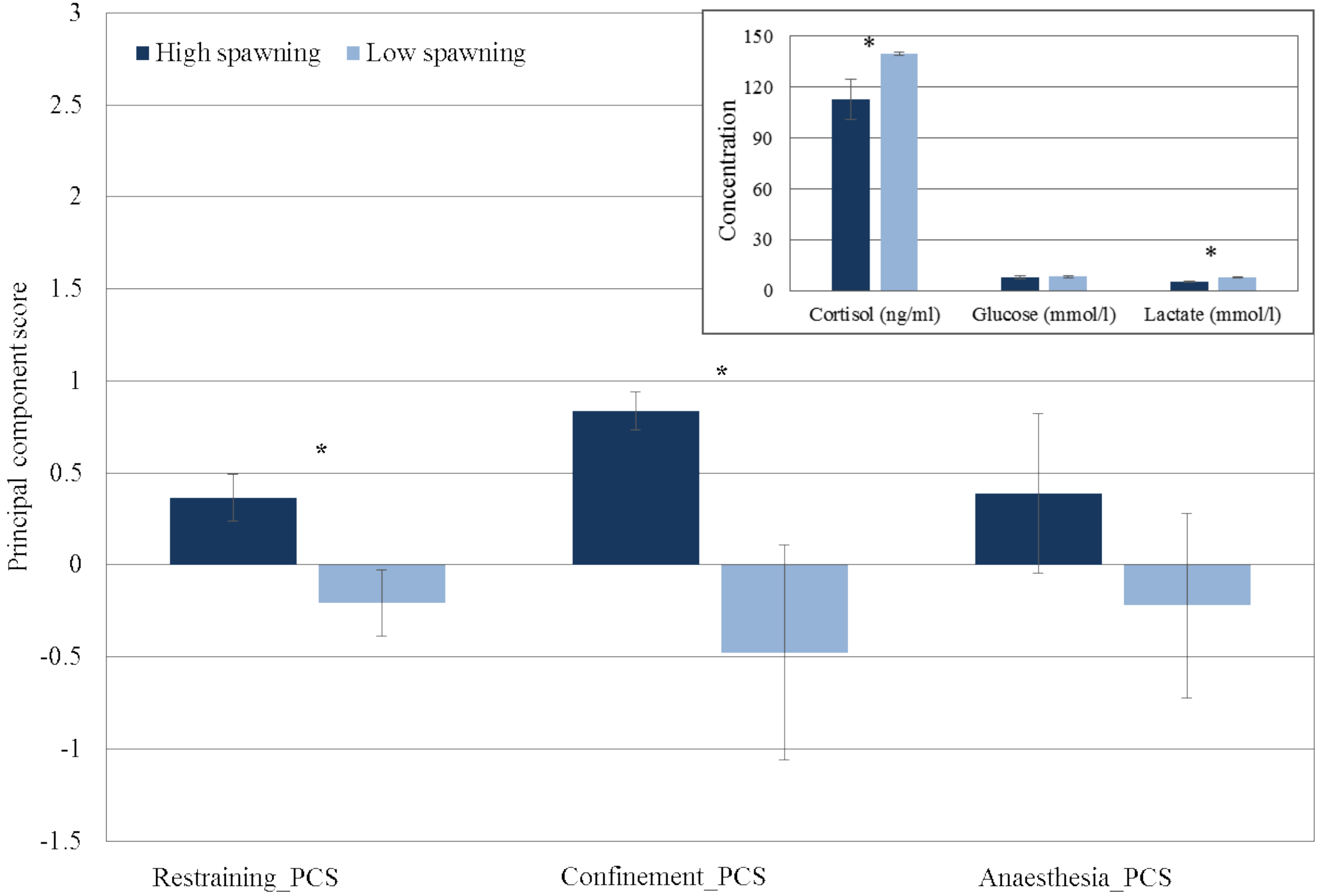

Crossed
Did not cross
Principal component score
2.5
2
1.5
1
0.5
0
-0.5
-1
*
*
Restraining_PCS
Confinement_PCS
Anaesthesia_PCS
Conentration
200
160
120
80
40
0
*
*
Cortisol (ng/ml)
Glucose (mmol/l)
Lactate (mmol/l)

Figure 3.


A
R² = 0.914
P = 0.001
NetFirstAct_trial 1
NetFirstAct_trial 2
B
R² = 0.746
P = 0.001
NetTotAct_trial 1
NetTotAct_trial 2
D
R² = 0.685
P = 0.001
ConLat_trial 1
ConLat_trial 2
C
R² = 0.413
P = 0.048
NetEsc_trial 1
NetEsc_trial 2
E
R² = 0.898
P = 0.001
ConAct_trial 1
ConAct_trial 2
F
R² = 0.541
P = 0.009
ANE3_trial 1
ANE3_trial 2

769 **Figure 4.**



770

Figure 5.

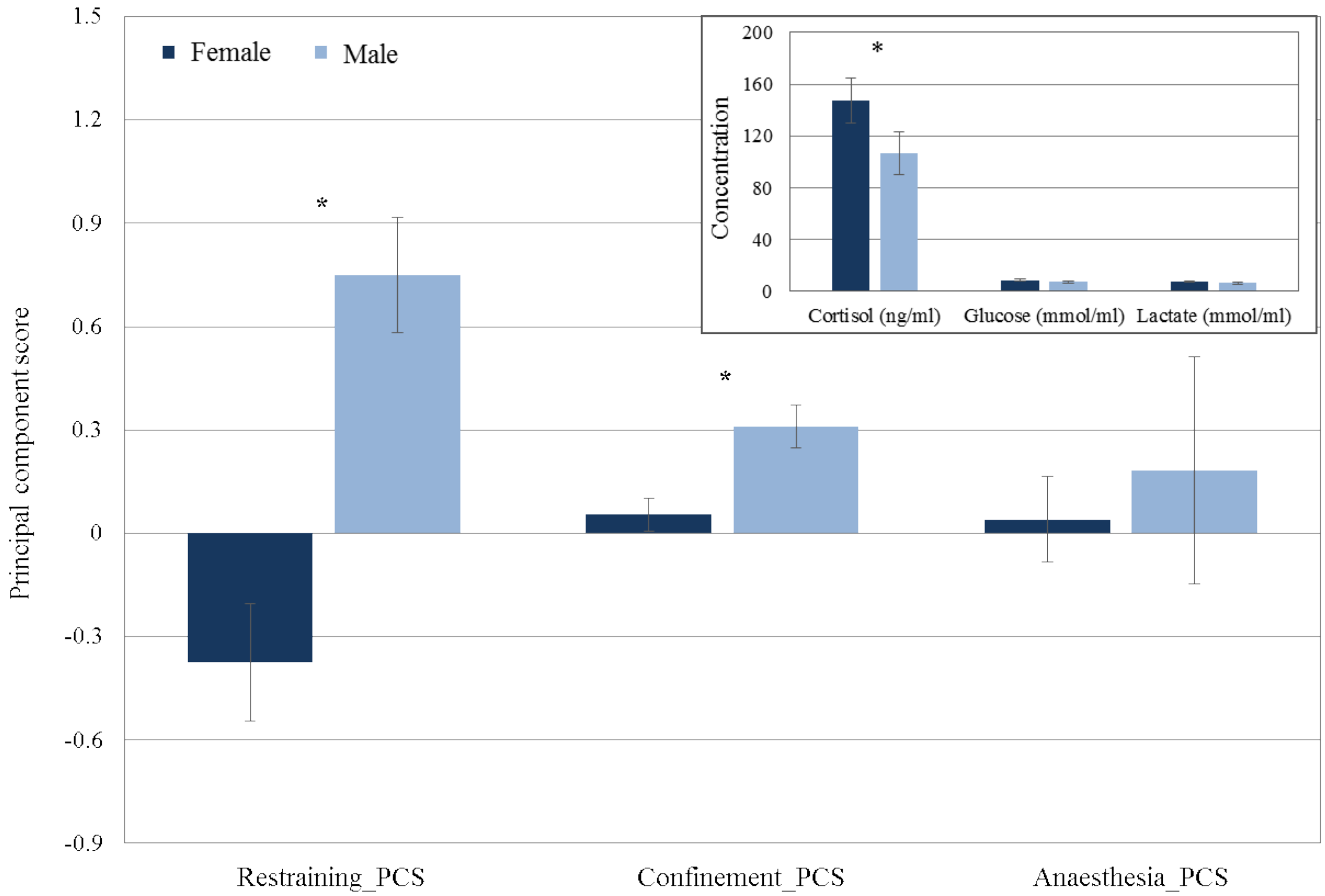
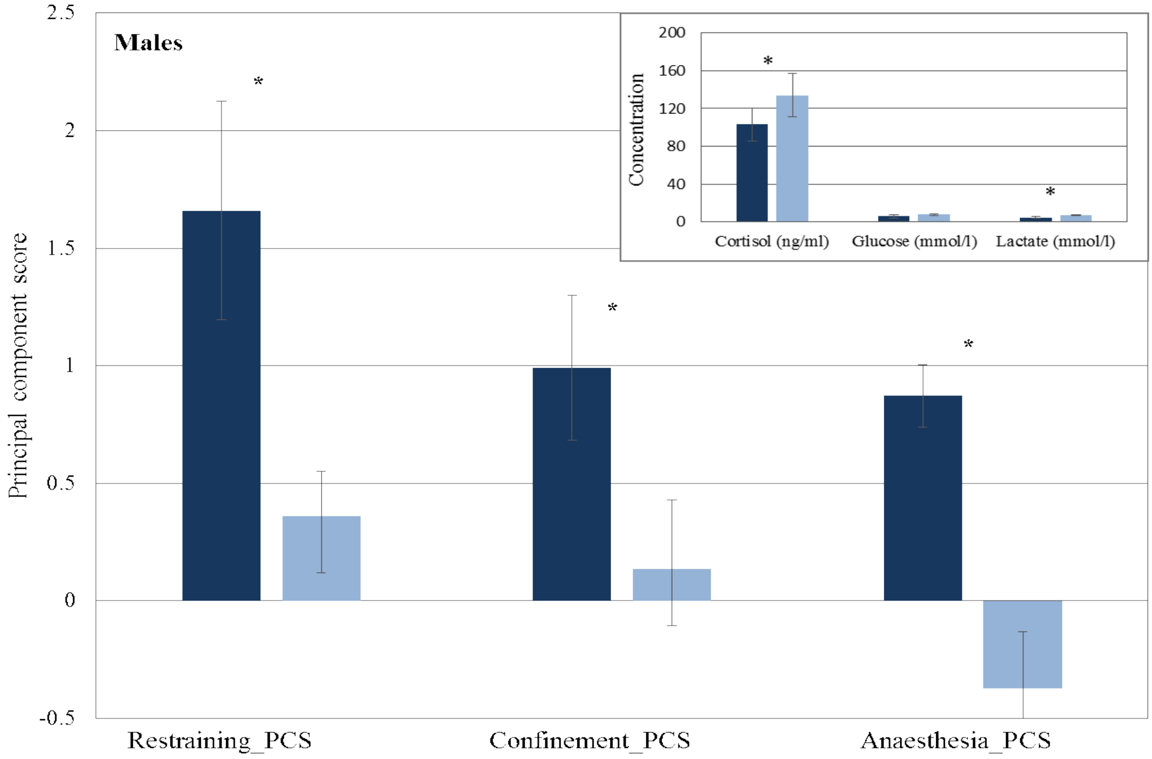
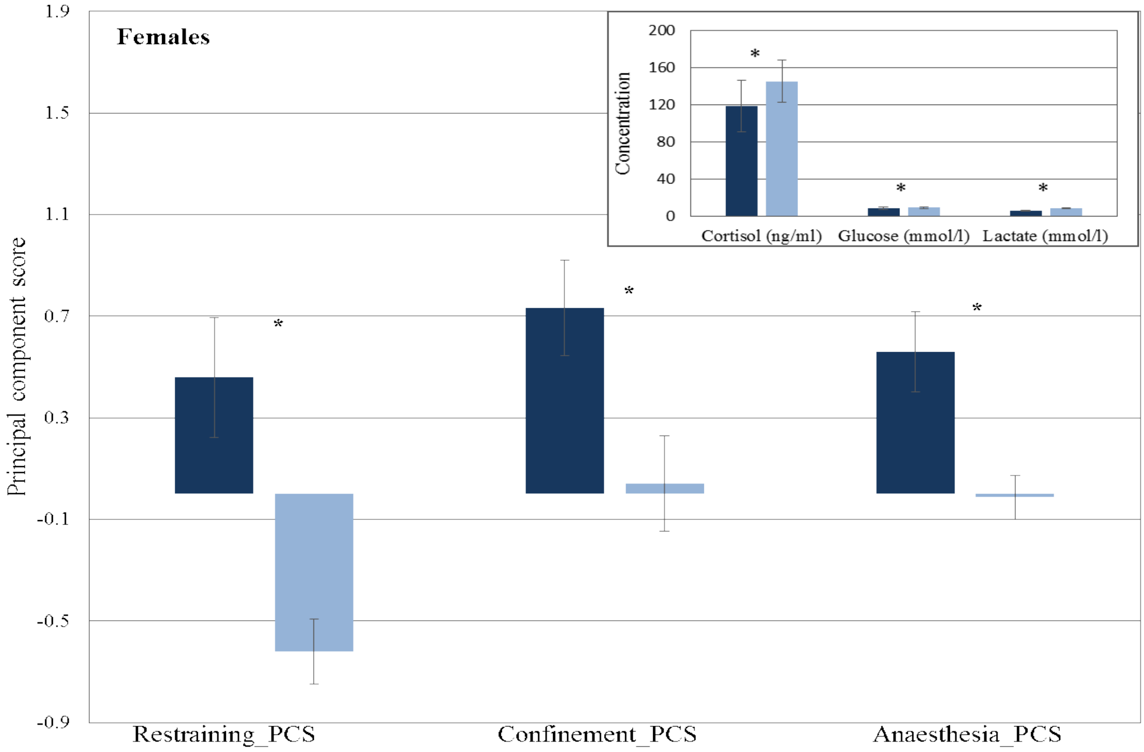

Female
Male
1.5
1.2
0.9
0.6
0.3
0
-0.3
-0.6
-0.9
Principal component score
*
*
Restraining_PCS
Confinement_PCS
Anaesthesia_PCS
200
160
120
80
40
0
Concentration
*
Cortisol (ng/ml)
Glucose (mmol/ml)
Lactate (mmol/ml)

Figure 6.


Males
Principal component score
2.5
2
1.5
1
0.5
0
-0.5
*
*
*
Restraining_PCS
Confinement_PCS
Anaesthesia_PCS
Concentration
200
160
120
80
40
0
*
*
Cortisol (ng/ml)
Glucose (mmol/l)
Lactate (mmol/l)


Females
Principal component score
1.9
1.5
1.1
0.7
0.3
-0.1
-0.5
-0.9
*
*
*
Restraining_PCS
Confinement_PCS
Anaesthesia_PCS
Concentration
200
160
120
80
40
0
*
*
*
Cortisol (ng/ml)
Glucose (mmol/l)
Lactate (mmol/l)