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Links between reproduction and immunity in two sympatric wild marine fishes

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Abstract

According to life-history theory, limited resources can result in trade-offs between costly physiological functions. Particularly, it can be expected that individuals present lower immune function, or an alternative immunological strategy, during their reproductive compared to their non-reproductive season. Here we investigate the link between reproduction and immunity in two sympatric marine fish species, the rockfish *Sebastes oculatus* and the sandperch *Pinguipes brasilianus.* The results showed lower values of total white blood cells and spleen index, but higher levels of natural antibodies (only in females) in reproductive rockfish compared to non-reproductive ones. On the other hand, reproductively active sandperch showed lower levels of natural antibodies and a higher neutrophil to lymphocyte ratio and spleen index (only in males), compared to non-reproductive ones. Also, negative correlations between reproductive and immune parameters were observed in female rockfish at the individual level, but not in sandperch. Our results are consistent with the presence of different immunological strategies in reproductive and non-reproductive periods, with patterns that appear to be species-specific.

This specificity suggests that various aspects of immunity might respond differentially to resource limitation, which could be associated with the disparate life-history strategies of the studied species. Alternatively, though not exclusively, the observed patterns could be driven by abiotic factors that characterize the reproductive season of each species (i.e., winter for rockfish, summer for sandperch). Our study contributes to ecoimmunological knowledge on free-living fish and highlights that detection of trade-offs can depend on the combination of study species, season, sex, and specific immune components measured.

Key words: ecoimmunology, marine fish, innate immunity, reproductive hormones, trade-off.

1. Introduction

Immune function provides defense against parasites and diseases to maximize individual fitness (reproductive output and survival) (Roitt et al., 1998). Among wild animals, immune responses can vary along organismal life, with several different, and potentially interacting, factors driving these variations (Martin et al., 2008; Madelaire et al., 2021). Accordingly, a primary goal of ecoimmunology has been to describe and explain variation in immune function (Sheldon and Verhulst, 1996; Kernbach et al., 2019), particularly why and how extrinsic and intrinsic factors contribute to shape immunity in free-living organisms (Martin et al., 2008). Intrinsic factors such as age, sex, reproductive or nutritional status, as well as extrinsic factors such as environmental parameters (e.g., temperature, photoperiod), social position, or geographic location, can modulate immune responses (Zapata et al., 1992; Nelson and Demas, 1996; Bowden et al., 2007; Martin et al., 2008). Thus, an interplay among intrinsic and extrinsic factors determines the capacity of individuals to control disease and eventually influence survival (Madelaire et al., 2021). Central to ecoimmunology is the notion that immunity is costly, entailing costs in terms of energy, amino acids, and/or micronutrients (Sheldon and Verlhust, 1996; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000). Consequently, trade-offs are expected to occur between immune function and other key physiologically demanding processes such as growth or reproduction. Variation in immunity could thus result from its relative benefits and costs in different contexts (Martin et al., 2008; Tieleman, 2018).

Immunity and reproduction are two central aspects of individual fitness, both being physiologically and resource demanding (Rauw, 2012; immune cost examples: reviewed by Lochmiller and Deerenberg, 2000; reproductive cost examples: reviewed by Edward and

Chapman, 2011). How these two functions are traded-off has attracted the attention of researchers (Downs and Stewart, 2014). Although organisms can sometimes mitigate the elevated costs of reproduction and immunity by increasing resource intake (Ruiz et al., 2010), resources are typically limited in natural environments and thus must generally be distributed among competing physiological processes (Sheldon and Verhulst, 1996; Norris and Evans, 2000; Brace et al., 2017). Findings of lower immunity in reproductive versus non-reproductive individuals in multiple taxa are consistent with allocation of limited resources to reproduction at the expense of immune function (Krams et al., 2017; Palacios and Bronikowski, 2017; Durso and French, 2018; Hayward et al., 2019). Although the physiological mechanisms underlying the trade-off between the reproductive and immune systems are still unclear, some studies in vertebrates suggest that sex steroid hormones could play a role as mediators (Klein, 2000a,b; Martin et al., 2008; Segner, 2016; Shepherd et al., 2021). For example, estrogens and androgens can modulate immune functions through molecular and cellular mechanisms, which could account for the observed differences in immunity between reproductive and non-reproductive individuals (Milla et al., 2011; Campbell et al., 2021). Estrogens generally enhance both cell-mediated and humoral immune responses; however, there are reports of estrogens suppressing some cell-mediated functions (Klein, 2000b; Campbell et al., 2021). On the other hand, androgens are typically, but not always, immunosuppressive (Klein, 2000b; Campbell et al., 2021).

From an ecoimmunological point of view, the relationship between immune and reproductive functions in vertebrates has been mainly examined in endotherms, particularly birds (reviewed by Martin et al., 2008; Valdebenito et al., 2021). Studies in ectotherms, and particularly in fish, have been less common. Teleost fishes are the largest and most diverse group of vertebrates, consisting of more than 33,000 species living across all aquatic environments (Nelson et al., 2016) and accounting for approximately 50% of the extant vertebrates (Trudeau, 2018). This group has significant economic, social, and environmental impacts worldwide (Pikitch et al., 2014; Lynch et al., 2016; Trudeau, 2018). Despite their broad diversity and global importance, ecoimmunological studies in teleosts have so far focused primarily on a relatively small group of boreal and/or commercially important freshwater species such as salmonids and cyprinids. Among those studies, however, the few in free-living fishes generally support the existence of a trade-off between reproduction and immune function (Skarstein et al., 2001; Sana et al., 2002; Kortet et al., 2003; Rohlenová and Šimková, 2010; Lamková et al., 2007) and set the stage for research in other free-living species, including studies involving more than one species to gain insight into other interesting aspects of these trade-offs, such as potential species-specificity.

In the present study, taking advantage of previously published immunological (Sueiro and Palacios, 2016; Sueiro et al., 2020) and reproductive (Sueiro et al., 2022) data, we investigated the link between immunity and reproduction in two sympatric marine fish species: the rockfish *Sebastes oculatus* Valenciennes, 1833 (Scorpaeniformes: Sebastidae) and the Brazilian sandperch *Pinguipes brasilianus* Cuvier, 1982 (Perciformes: Pinguipedidae). These species inhabit temperate reefs of the northern Patagonian gulfs, which are small rocky patches scattered over large extensions of sandy, gravel and limestone seabeds (Ciocco, 1988). Rocky reefs shelter low-diversity fish assemblages, with rockfish and sandperch being two of the most abundant and commonly found species (Galván et al., 2009; Irigoyen et al., 2013). Both are long-lived top demersal predators on the benthic food web showing high site fidelity to shallow and rocky substrates (Galván et al., 2009; Irigoyen et al., 2013). Both species are gonochoristic but differ in reproductive strategy, being *S. oculatus* a viviparous species and *P. brasilianus* an oviparous one.

To achieve our goal, we analyzed available data on different aspects of innate immunity (total white blood cells, spleen somatic index, bacterial agglutination, and neutrophil to lymphocyte ratio) and reproductive parameters (steroid hormone plasma levels and gonadosomatic index) of individuals in reproductive and non-reproductive condition from both species and sexes. Based on the hypothesis of costs of reproduction on immune function we predicted: (1) lower immune function in reproductive than non-reproductive fish of both species, although not all immune parameters might be equally affected, (2) negative relationships between reproductive and immune parameters across individuals within each species, present exclusively, or more markedly, during the reproductive season, and (3) potentially different patterns of association between reproductive and immune parameters for the two study species.

2 Material and Methods

2.1. Ethical procedures

Field work and samples of fish were authorized by the Secretaría de Turismo y Áreas Protegidas and Secretaría de Pesca del Chubut (Disp 156-SsCyAP/17). Fish collection and euthanasia was carried in accordance with the Guidelines for the Use of Fishes in Research of American Fisheries Society-American Institute of Fishery Research Biologists-American Society of Ichthyologists and Herpetologists.

2.2. Study species

Rockfish is a viviparous species, reproductively active during the austral winter-early spring (June-November) and inactive during the austral summer-autumn (December-May). Its reproductive cycle is annual with a synchronous cycle for males and females and gestation lasts about one month with parturition occurring towards the end of spring (Marcinkevicius, 2019). In contrast, sandperch is an oviparous species with an extended reproductive season encompassing spring and summer (October-February) followed by a recovery period during colder months (March-August) (Villanueva-Gomila et al., 2015).

2.3. Field site and fish sampling

The data used in this work were collected as part of a series of studies assessing the effect of water pollution on fish physiology (Sueiro and Palacios, 2016; Sueiro et al., 2020; 2022). For the present study, only data from individuals sampled at reference sites (i.e., not exposed to contamination) were considered. Below we provide a brief description of the original fieldwork involved. Adult rockfish (n = 96; Females: 63, Males: 33) and sandperch (n = 67; F: 49, M: 18) were hand-lined or spear-fished on winter months July-August 2013-2016 (seawater temperature 9 ± 0.5 ºC) and summer months January-March 2014-2017 (temperature 18 ± 0.5 ºC). Theses sampling times include the reproductive season, when the highest proportion of individual are in spawning phase (i.e. gonads exhibiting developing or mature stages) and the non-reproductive season when the highest proportion of individual are in resting phase (i.e. gonads exhibiting recrudescing and early developing phase) (details in Sueiro et al., 2022). Fish were caught from shallow rocky reefs (Punta Este - 42.47°S, 64.56°W; Cerro Avanzado - 42.50ºS, 64.52ºW; and Bañuls - 42.39°S, 64.59°W) located in Nuevo Gulf (Chubut province, Argentina). Sampling times ranged between 3 and 5 minutes, a range below the times for which an acute stress could affect immune variables (Guo and Dixon, 2021). Blood samples were collected, and plasma used for immune and reproductive hormone analyses. Total body mass (TBM, g), total length (TL, cm), spleen mass (g), and gonadal mass (GM, g) of individual fish were recorded. Sex identification was made by internal examination of the gonads. Except for different morphology in the genital papillae of *S. oculatus* males and females (Marcinkevicius, 2019), there is no other external sexual dimorphism in the fishes studied.

2.3. Reproductive and immune parameters

Protocols for reproductive parameters have been recently described by Sueiro et al., (2022). Briefly, total plasma androgens (A) and 17β-estradiol (E_2) levels were measured using a validated radioimmunoassay (RIA) system (Elisio et al., 2019). The gonadosomatic index (GSI), which provides information about maturation and gonad development (Flores et al., 2019), was calculated as the percentage of gonad mass (GM) relative to body mass without gonads.

All protocols for immune parameters have been described in detail by Sueiro and Palacios (2016) and Sueiro et al. (2020). Briefly, total white blood cell counts (total WBC), which are part of the cellular immune system and reflect the general state of health and immune response of fish (Seibel et al., 2021), were estimated by counting all leukocytes in ten consecutive microscope fields at 400×. Natural antibodies are nonspecific immunoglobulins produced constitutively and one of their many functions is agglutination- of foreign cells leading to pathogen neutralization (Ochsenbein and Zinkernagel, 2000). Bacterial agglutination mediated mostly by natural antibodies present in plasma was determined via an agglutination assay in 96-well plates using *Escherichia coli* (ATCC 8739) that we had previously adapted for our study species. The spleen-somatic index (SSI) was calculated as spleen mass (g)/body mass (g) × 100, where body mass was the mass of the fish without gonads. Spleen size is widely used as a simple measurable immune parameter with a potential role in the immune response against parasite infection (Kortet et al., 2003; Rohlenová et al., 2011). The neutrophil to lymphocyte ratio (N:L), which provides information on immune defense strategy and is considered an index of stress in vertebrates (Davis et al., 2008), was calculated from the percentage of neutrophils and lymphocytes estimated from blood smears by classifying the first 200 leukocytes encountered under 1000× magnification. Additionally, hematocrit (HCT) and condition factor (CF) were considered as global indicators of fish health to assess whether study individuals were overall healthy. Hematocrit (HCT) was measured as the percentage of packed red blood cells in the total blood volume after blood centrifugation for 5 min at 12000 g in heparinized capillary tubes. The condition factor was calculated as CF= (100 x M)/TL^3, where M is the mass of the fish without gonads in grams and TL is the total length of the fish in centimeters (Goede and Barton, 1990).

2.4. Statistical analyses

Reproductive (E_2, A, and GSI), immune (WBC, SSI, BA, and N:L), and health-related (HCT and CF) parameters were examined using linear models (Crawley, 2013) that included the explanatory effects of the reproductive season (reproductive or non-reproductive), sex (male or female), their interaction, and total body length (TL) as covariate in order to control for variation in individual size/age. Species were analyzed separately. In both species, TL did not differ with sex, season, or their interaction (all P >

0.22). In the case of significant season by sex interactions, post-hoc comparisons of estimated marginal means were performed. Sample sizes differ among analyses because not all measurements could be obtained for some individuals. To meet statistical assumptions of linear models, some parameters were transformed before analyses. 17β-estradiol, N:L, and CF were square rooted, A and total WBC were log-transformed, GSI was analyzed as log (GSI + 0.05) and SSI as the square root of (SSI + 0.01). For each parameter, we report the coefficient of determination (R^2) obtained from the best models (i.e., models including significant predictors only).

In addition to the linear models, we used path analyses to gain insights into how the reproductive and immune variables were related within females of each species (males were not analyzed due to low sample sizes). Path analyses allow the quantification of the relative influence and the significance of relationships among variables using models that specify a priori the directionality of potentially causal relationships (Olobatuyi, 2006). Four path analyses were conducted, as reproductive and non-reproductive females of each species were analyzed separately to avoid the potential confounding of the season on individual-level relationships. Considering the limitations imposed by sample size that preclude the evaluation of complex paths, we evaluated concise models that included the GSI and the main female reproductive hormone (E_2) and the subset of immune variables showing significant effects of reproductive vs. non-reproductive season in our linear model analyses. All path analyses were performed on standardized variables, so estimates are standardized coefficients. In variables correlated with TL, we used the residuals from the regression of the variable on TL to control for this variation. Statistical analyses were carried out using R software, ver. 4.2.0 (R Development Core Team, 2022). We evaluated model fit using DHARMa package (Hartig, 2022). Comparisons of estimated marginal means from interactions in linear models were made using emmeans package (Lenth, 2022). Path analyses were performed using the LAVAAN package (Rosseel, 2012) in R. All tests were two-tailed, and differences were considered significant at $P < 0.05$.

3. Results

3.1. Reproduction and immunity of the rockfish Sebastes oculatus

Reproductive parameters of females and males differed significantly between the reproductive and non-reproductive seasons (Table 1). Specifically, females in the reproductive season had higher levels of E_2 (predicted mean ± SE from models; Reproductive Females (RF): 0.96 ± 0.02; Non-reproductive Females (NRF): 0.65 ± 0.02; $t = 2.6$, $P = 0.012$), A (RF: -0.57 ± 0.03; NRF: -1.65 ± 0.03; Table 1), and GSI (RF: 1.95 ± 0.02; NRF: -0.79 ± 0. 03; $t = 20.5$, $P < 0.001$) than non-reproductive ones. Males, on the other hand, with the exception of E_2 levels that were similar in both seasons (Reproductive Males (RM): 0.43 ± 0.03; Non-reproductive Males (NRM): 0.56 ± 0.03; $t = 0.7$, $P = 0.470$), exhibited higher A levels (RM: -1.15 ± 0.04; NRM: -2.28 ± 0.04; Table 1) and GSI (RM: -0.24 ± 0.06; NRM: -1.80 ± 0.04; $t = 8.3$, $P < 0.001$) during the reproductive season compared to the non-reproductive season.

Regarding immune function, season had an effect on total white blood cell counts (total WBC), spleen somatic index (SSI), and bacterial agglutination (BA) (Table 1; Fig. 1A-C). Both sexes exhibited lower total WBC and SSI during the reproductive season (Table 1; Fig. A and C, respectively); during this season, females also showed higher BA ($t = 3.7$, $P < 0.001$, Fig. 1B). On the other hand, neutrophil to lymphocyte ratios (N:L) were not affected by season (Fig. 1D; Table 1). Finally, general-health status (i.e., hematocrit and condition factor) was not affected by season either in females or males (Fig. 1E and F; Table 1). Total length (TL) had a positive effect on GSI and CF, while its effect on total WBC and SSI was negative (Table 1).

3.2. Reproduction and immunity of the sandperch Pinguipes brasilianus

Females exhibited higher E_2 levels (predicted mean ± SE from models; RF: 0.31 ± 0.01; NRF: 0.18 ± 0.01; *t* = 3.5, P = 0.001) and GSI (RF: 1.12 ± 0.01; NRF: 0.29 ± 0.01; Table 2) during the reproductive than the non-reproductive season, whereas their A levels were similar across seasons (RF: -1.40 ± 0.01; NRF: -1.27 ± 0.01; Table 2). Males showed higher GSI during the reproductive season (RM: -0.43 ± 0.01; NRM: -1.29 ± 0.01; Table 2), while neither their E_2 nor A levels differed between seasons (E_2 = RM: 0.13 ± 0.01; NRM: 0.16 ± 0.03; A= RM: -1.46 ± 0.02; NRM: -0.40 ± 0.01; Table 2).

Among the immune parameters, season influenced BA, SSI, and N:L ratio (Table 2; Fig. 2B, C, D, respectively). Reproductive males showed higher SSI than non-reproductive individuals (*t* = 2.8, P = 0.006, Fig 2C) and both reproductive females and males exhibited lower BA (Table 2; Fig. 2B) and higher N:L ratios (Table 2; Fig. 2D) than individuals sampled during the non-reproductive season. Total WBC did not show differences between seasons (Table 2, Fig. 2A). Regarding general-health status, neither hematocrit nor condition factor varied with season (Table 2, Fig. 2E and F). Total length (TL) had no effect on any of the parameters measured (Table 2).

Individual-level relationships between reproductive and immune parameters

Path models were constructed considering predictor variables that showed significant season effects for females in our linear model analyses. Thus, hypothesized model paths for rockfish females included: (a) the influence of E_2 on GSI, (b) the influences of E_2 and GSI on BA, (c) the influences of E_2 and GSI on total WBC, and (d) the influence of GSI on SSI (Fig. 3A). For sandperch females, hypothesized model paths included: (a) the influence of E_2 on GSI, (b) the influences of E_2 and GSI on BA, and (c) the influences of E_2 and GSI on N:L ratios (Fig. 3B).

We found that E_2 and GSI showed significant associations with few of the evaluated variables and did so only during the reproductive season for each species (Fig. 3C and D, Table I supplementary material). For rockfish, females showing higher E_2 levels had lower total WBC counts and females with higher GSI showed lower SSI (Fig. 3C); the remaining paths were not significant (Table I supplementary material). For sandperch, females having higher E_2 levels showed higher GSI (Fig. 3D), while the remaining paths were not significant (Table I supplementary material).

4. Discussion

In the present field study, we investigated the link between reproduction and immunity in two sympatric marine fish species inhabiting Patagonian waters. In accordance with our predictions, we found that (1) both species, rockfish and sandperch, showed a decrease in some, but not all, of the immunological parameters measured during the reproductive season relative to the non-reproductive season. In line with our second prediction (2), we found negative correlations between some reproductive and immune parameters at the individual level in female rockfish, but not sandperch, during the reproductive season. Finally, we found support for our third prediction (3) of variation between the two studied species in their pattern of association between immunity and reproduction. Overall, our results are consistent with the presence of different immunological strategies during reproductive and non-reproductive periods, with patterns that appear to be species-specific, suggesting that various aspects of immunity might respond differentially to resource limitation. In addition, we found a few differences between males and females in immune parameters that would be in line with sex-specific immune strategies. These results should be viewed with caution as we cannot rule out that the sex-related patterns are driven by the difference in sample sizes between the sexes.

4.1. Immune component-specific costs of reproduction

Studies involving wild and captive individuals have found evidence supporting the idea of trade-offs between reproduction and immune function. Such trade-offs, however, do not necessarily involve all aspects of immune function, suggesting that patterns can be immune component specific. For instance, Kortet et al. (2003) found that reproduction was associated with reduced spleen size and migration activity of head kidney granulocytes, but not with phagocytic activity of head kidney granulocytes, blood IgM concentration, or white cell counts in two natural populations of the cyprinid fish, *Rutilus rutilus*. Similarly, for Arctic charr, *Salvelinus alpinus*, lower spleen mass was found in spawning males compared to resting individuals, while no differences were observed in granulocyte and lymphocyte densities (Skartein et al., 2001). Our study in two sympatric marine species supports the notion of immune component-specific trade-offs with reproduction. The high GSI and gonadal steroid hormone levels exhibited by reproductive rockfish were accompanied by depressed total WBC and lower SSI, whereas N:L ratios were no different from those of non-reproductive individuals. On the other hand, reproductively active sandperch, showing high GSI and/or reproductive hormone levels, had overall lower BA titers and higher N:L ratios, but no differences in total WBC compared to non-reproductive fish. This immune component-specificity suggests that different aspects of immunity respond in different ways to the challenges imposed during the breeding season, which is reasonable due to the need for organisms to maintain immune surveillance during resource demanding life-history stages to prevent infections and facilitate survival (Graham et al., 2011).

4.2. Reproductive-immune associations in the rockfish Sebastes oculatus

A decrease in total WBC, as observed in this study for reproductive rockfish, is consistent with immunodepression of the cellular components during reproduction. Moreover, for reproductively active females, we also found a negative relationship between E_2 levels and total WBC at the individual level, suggesting a potential mechanism for this trade-off. Steroid hormones can modulate both innate and adaptive immune responses in addition to reproductive function (Segner et al., 2007; Verburg-van Kemenade et al., 2016) and sex steroid receptors are present in many immune tissues and cells (Tokarz et al., 2015; Burgos-Aceves et al., 2017). Steroid-immune interactions have been reported in many fish species (Harris and Bird, 2000; Szwejser et al., 2017; Campbell et al., 2021). For instance, in rainbow trout (*Oncorhynchus mykiss*) (Hou et al., 1999) and gilthead seabream (*Sparus aurata*) (Cuesta et al., 2007), the rise of E_2 levels is correlated with a decrease of plasma IgM levels. Similarly, Watanuki et al., (2002) reported that E_2 injection has a suppressive effect on head kidney phagocytic cells of common carp (*Cyprinus carpio*). On the other hand, recent studies in female *Channa punctata*, have shown that the highest expression of

NOD-like receptors, which play a key role in inflammation, coincides with the peak estradiol level during the reproductively active phase (Chuphal et al., 2023). In general, however, low levels of E_2 stimulate immune responses, while elevated E_2 levels appear to be rather immunosuppressive (Szwejser et al., 2017), which is in line with our finding for female rockfish. Our results, together with the knowledge of the integrative role these hormones play in mediating reproductive activity and immunomodulation, suggest that E_2 might be the physiological mediator of the immune-reproductive trade-off underlying changes in white blood cell counts of female rockfish.

Reproductive rockfish also showed a low SSI compared to non-reproductive individuals. In teleosts, the spleen is a principal lymphomyeloid tissue and plays an important role in haematopoiesis, antigen degradation, and antibody production (Zapata et al., 1996). Decreased spleen size during the breeding season has also been reported in other wild fish populations such as those of the salmonid Arctic charr (*Salvelinus alpinus)* (Skarstein et al., 2001), and cyprinids as the roach (*Rutilus rutilus)* (Kortet et al., 2003) and the chub (*Leuciscus cephalus*) (Lamoková et al., 2007). In addition to the difference in SSI between reproductive and non-reproductive rockfish, path analysis revealed that during the breeding period, the increase in gonad mass of female rockfish, reflected by a higher GSI, was negatively correlated with SSI. This suggests that higher investment in reproductive function results in lower investment in immune defense, as predicted by the trade-off hypothesis. Similarly, Šimková et al. (2008) found a negative correlation between spleen and gonadal sizes in cyprinid females, which the authors interpreted as evidence of an energetic trade-off between reproduction and immunity. Alternatively, a large spleen size in fish (which in our study was observed for non-reproductive individuals) can be interpreted as an indication of high immunological activity from already established infection (Skarstein et al., 2001). In our study the latter explanation seems unlikely given that reproductive and non-reproductive individuals did not vary in general-health status, at least as measured through the condition factor and hematocrit.

Contrary to the idea of a reproductive-immune trade-off, we found for females (but not males) that BA was higher during the reproductive season. This humoral aspect of innate immunity indicates the ability of plasma agglutinins, such as natural antibodies, to agglutinate bacteria (Alvarez-Pellitero, 2008). Natural antibodies, which are mostly of the IgM isotype, can recognize a broad array of pathogens (e.g., bacteria, viruses) and are involved in early resistance against infection, constituting an important nexus between innate and acquired immune responses (reviewed by Ochsenbein and Zinkernagel, 2000). Similar to our result for female rockfish, Swain et al. (2007) reported higher titers of bacterial agglutination during the reproductive period (rainy season) in Indian major carp (*Labeo rohita)*. Other studies have also reported an increase in immunological aspects concomitant with reproduction in fish. For example, Saha et al. (2002) found that IgM levels were higher in breeding common carp and Rohlenová and Šimková (2010) found higher respiratory burst and leukocyte counts during the spawning season of the cyprinid (*Leuciscus cephalus*). In our study, the higher BA during the reproductive season was only detected in females. Sex-related variation in immune responses has been documented in different fish species (reviewed by Campbell et al., 2021), with steroid hormones (Bakshi and Rai, 2020) and/or maternal transfer of immune factors (Swain and Nayak, 2009) as potential modulators of such variation. It has been proposed that in viviparous species such as rockfish, sex-specificity in immunity could be related to the need of females to maintain a higher level during vitellogenesis and oogenesis for maternal transfer, which is important for reducing mortality at larval/post larval stages (Swain and Nayak, 2009).

Finally, it is worth mentioning that for both sexes, lower total WBC and SSI during the reproductive season were accompanied by higher A levels. This could suggest a negative influence of A on these parameters. In this sense, androgens have long been considered immunodepressive in vertebrates (Martin et al., 2008). However, results in fishes, mainly salmonids and cyprinids, are mixed, with some studies showing negative effects, others positive, and others no effect of androgens on immunity (Harris and Bird, 2000; Campbell et al., 2021). Thus, the influence of androgens, as well as estrogens (Chuphal et al., 2023), seems to depend on the fish species, the steroid dose, and the immunological parameter studied.

4.3. Reproductive and immune associations in the sandperch Pinguipes brasilianus

Female and male sandperch showed lower BA during the reproductive compared to the non-reproductive season, suggesting that this immune aspect might trade-off with reproduction in this species. This is contrary to our observation for female rockfish and for the Indian major carp (Swain et al., 2007). Relatively few studies in fish have examined seasonal variation in bacterial agglutination. However, studies on teleost IgM concentrations in serum (presumably also representing levels of natural antibodies) show that patterns differ across species, with some fishes showing higher values during the reproductive period (e.g., Suzuki et al., 1996; Hou et al., 1999), some showing the inverse (Suzuki et al., 1997; Kortet et al., 2003), and some showing no clear relationship with reproduction (Rohlenová et al., 2011).

In addition to the breeding-related reduction in BA, N:L ratios increased in both female and male reproductive sandperch. Similarly, studies in other vertebrate species including birds (Palacios et al., 2018) and reptiles (Madelaire et al., 2021) have reported increases in N:L ratios during the reproductive season. Neutrophils and lymphocytes are white blood cells involved in the immune response (Campbell and Ellis, 2007), and changes in their ratio are indicative of a switch in immune defense strategy (Davies et al., 2008; Beldoménico et al., 2008). The N:L ratio has also been considered an index of stress in vertebrates (Davis et al., 2008). The multiple causes of alteration in numbers of circulating neutrophils and lymphocytes, however, make determining the cause of changes in N:L ratio a challenge; therefore, interpretation must be made in context with other parameters measured (Davis et al., 2008). In our study, the observed increment of N:L ratios in reproductive sandperch resulted from a reduction in the proportion of circulating lymphocytes together with an increase in the proportion of circulating neutrophils. Thus, the variation in N:L ratios could be indicative of immune-redistribution, an alteration in leukocyte 'trafficking' between peripheral blood and other body compartments (Dhabhar et al., 1994; 1995). In this sense, reproductive sandperch could be altering their immune strategy through the decrease of lymphocyte-mediated defenses in blood, which would be in line with the observed reduction in natural antibody levels (measured via the bacterial agglutination assay) that are produced by lymphocytes. Alternatively, given that reproduction is the most metabolically demanding activity in the lives of fishes (McBride et al., 2015), the high N:L ratio could be a sign of stress driven by the high energy demand imposed by reproduction. Despite the observed immune variation linked to sampling season in sandperch, no individual-level associations between immune (i.e., BA and N:L ratios) and reproductive parameters were found for reproductive females in our path analyses. Although several authors have reported immune alterations in parallel with changes in reproductive parameters in wild fishes (e.g., across months or seasons), finding such associations at the individual-level (i.e., significant correlations) seems to be more complex and to depend on sex, species, sensitivity of immune factors, and steroid hormone concentrations (Hou et al., 1999; Saha et al., 2002; Vainikka et al., 2004; Rohlenová et al., 2011).

Finally, we found that males in the reproductive season (but not females) exhibited higher SSI compared to non-reproductive individuals. This result contrasts with the one for rockfish, described earlier, as well as those previously reported for other fish species (Karstein et al., 2001; Kortet et al., 2003). Other studies in male fish, however, have reported an increase in some immune parameters during reproduction. For instance, Rohlenová and Šimková (2010) found that respiratory burst and leukocyte counts were higher in breeding males of the cyprinid *Leuciscus cephalus* relative to non-breeders. The authors argued that such high values could be reflecting the extensive stress due to spawning or, alternatively, may be induced by pathogens such as protozoa or viruses. In our study, given the small sample size for SSI in non-reproductive males, the pattern would need to be investigated further and confirmed before proposing potential causes.

4.4. Immunological variation and seasonality

Annual variation in immune function has been widely reported for fish species (Bowden et al., 2007; Ahmed et al., 2020). While variation has mostly been discussed regarding seasonal changes in abiotic factors, less attention has been given to the reproductive status of individuals (Campbell et al., 2021). Given that our two study species reproduce during different seasons of the year, seasonality needs to be considered in the interpretation of our results. Rockfish are reproductively active during winter (when seawater temperature is 9 ± 0.5 ºC) and non-reproductively active during summer (temperature 18 ± 0.5 ºC), while the opposite is true for sandperch. Considering that winter has generally been proposed as the immunosuppressive season (Bowden et al., 2007), major effects on reproductive trade-offs could be expected in rockfish than sandperch. Accordingly, the observed decrease in total WBC and SSI in rockfish during the reproductive season, besides being influenced by reproductive status, could also be driven by environmental factors that characterize the winter season (i.e., low water temperatures, short photoperiod, and decreased food availability). Contrarily, for sandperch each season would impose different challenges: summer regarding reproduction and winter regarding environmental conditions. During winter, however, except for the reduction in SSI (observed only for males and having a low sample size), no other immunological parameter decreased in this species.

Studies carried out both in wild and in captive fish have generally reported a decrease in immune parameters during winter, including spleen sizes (Rohlenová et al., 2011; Gradil et al., 2014), total white blood cells (Pascoli et al., 2011; Rohlenová et al., 2011; Papežíková et al., 2016), and natural antibodies (Suzuki et al., 1997; Kortet et al., 2003; Swain et al., 2007; Rohlenová et al., 2011), with water temperature generally considered as the strongest abiotic factor affecting immune function. Furthermore, studies have experimentally demonstrated the negative effect of cold-water temperatures (within the natural range for each species) on fish immune function (Ainsworth et al., 1991; Le Morvan et al., 1998; Alcorn et al., 2002; Aman and Khan, 2016; Abram et al., 2017).

Contrary to these general trends, we found that BA was higher during winter for both rockfish and sandperch. To our knowledge no other study has reported such pattern. This finding may indicate a more important defense role of natural antibodies and other agglutinins present in plasma during cold periods, when other immune components might become depressed. In this sense, this result could be in line with the notion that while acquired components are usually depressed by lower temperatures, some innate components might increase to offset this reduction (Le Morvan, 1998). Nevertheless, other measures of acquired immunity are needed to evaluate this hypothesis.

4.5. Species-specific association patterns between reproduction and immunity

The results discussed above show that these two sympatric wild marine fishes present different patterns of association between reproduction and immunity, with rockfish showing more evidence consistent with immune trade-offs with reproduction (even at the individual level) than sandperch. Although both species coexist in the same environment, widely overlapping in the use of resources such as food and refuge (Galván et al., 2009; Irigoyen et al., 2013), they differ not only in phylogenetic background but also in reproductive strategy. As mentioned earlier, the rockfish is a single-spawning viviparous species reproductively active during winter (Marcinkevicius, 2019), while the sandperch is a multispawning oviparous fish reproductively active in summer (Villanueva-Gomila et al., 2015). Thus, the documented differences between these species regarding their patterns of association between reproductive and immunological parameters could be driven by various factors (i.e., phylogeny, seasonality, reproductive mode) that cannot be disentangled in the present study. Related to reproductive modes, although in principle the energetic costs that oviparous and viviparous strategies encompass could influence how resources are allocated to different physiological processes, comparisons of energy requirements for reproduction between these two strategies are complicated and patterns not straight forward (McBride et al., 2015; Segner et al., 2017). In our study, potential differences in resource investment between viviparous rockfish and oviparous sandperch could contribute to the different patterns observed between species. However, evaluating this hypothesis will require further studies. In addition, the fact that rockfish are reproductively active during the most demanding season (i.e., winter), could be operating synergistically resulting in the detection of trade-offs, whereas this would not be the case for sandperch.

5. Conclusions

In summary, our study contributes to the field of ecoimmunology by presenting an evaluation of the links between reproduction and immunity in a relatively understudied group in this regard: free-living marine fishes. Our results involving two sympatric species and population- as well as individual-level analyses highlight how complex these relationships (and/or their detection) can be in free-living conditions. Although we found some evidence consistent with reproductive-immune trade-offs under natural settings, the complexity of our findings precludes generalizing across fish species and/or immune defense components. Rather, our results support the existence of different immune defense strategies during reproductive and non-reproductive periods and suggest that detection of trade-offs might depend on the combination of study species, season, sex, and specific immune components measured.

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Figure Captions

Figure 1. Immune, and general-health parameters of the rockfish *Sebastes oculatus* showing effects of season, sex and/or their interaction as predicted by linear models in Table 1. Dot plots show mean ,black dotted line), and 95% confidence intervals ,grey line) for each season ,reproductive and non-reproductive) and sex ,females and males). Sample sizes are depicted beneath the corresponding dots. Significant effects from models in Table 1 are shown in legend and for significant interactions, significant post-hoc comparisons are indicated by asterisks ,* = $P < 0.05$, ** = $P < 0.01$).

Figure 2. Reproductive, immune, and general-health parameters of the sandperch *Pinguipes brasilianus* showing effects of season, sex and/or their interaction as predicted by linear models in Table 2. Dot plots show mean ,black dotted line), and 95% confidence intervals ,grey line) for each season ,reproductive and non-reproductive) and sex ,females and males). Sample sizes are depicted beneath the corresponding dots. Significant effects from models in Table 2 are shown in legend and for significant interactions, significant post-hoc comparisons are indicated by asterisks ,* = $P < 0.05$, ** = $P < 0.01$).

Figure 3. ,A-B) Hypothesized path models showing the expected relationships between reproductive ,E_2 and GSI) and immunological parameters that showed significant effects in linear model analyses in Table 1 and 2 for ,A) rockfish females and ,B) sandperch females. ,C-D) Simplified path diagrams showing statistically significant relationships ,$P < 0.05$) between reproductive and immunological parameters in reproductively active ,C) rockfish females and ,D) sandperch females. Positive relationships ,+) are shown with grey arrows,

negative relationships ,-) with black arrows. See text for statistical details and Table I in supplementary material for full path analyses results.

Table 1. Linear models for immunological, reproductive, and health-state parameters of adult rockfish (*Sebastes oculatus*). All models included season (reproductive or non-reproductive), sex (male or female), and their interaction as predictor variables. Total body length (TL) was included as a covariate.

Parameter	Predictor	*F*	*P*
E_2 (ng/ml)	Season	$F_{1,70} = 0.75$	0.389
n = 75	Sex	$F_{1,70} = 5.99$	**0.017**
$R^2 = 0.168$	Season × sex	$F_{1,70} = 4.24$	**0.043**
	TL	$F_{1,70} = 2.68$	0.106
A(ng/ml)	Season	$F_{1,70} = 14.58$	**<0.001**
n = 75	Sex	$F_{1,70} = 3.52$	0.065
$R^2 = 0.190$	Season × sex	$F_{1,70} = 0.00$	0.974
	TL	$F_{1,70} = 1.04$	0.311
GSI	Season	$F_{1,90} = 344.2$	**<0.001**
n = 95	Sex	$F_{1,90} = 192.2$	**<0.001**
$R^2 = 0.872$	Season × sex	$F_{1,90} = 23.26$	**<0.001**
	TL	$F_{1,90} = 7.83$	**0.006**
WBC (n)	Season	$F_{1,74} = 17.53$	**<0.001**

n = 79	Sex	$F_{1,74} = 2.29$	0.135
$R^2 = 0.318$	Season × sex	$F_{1,74} = 1.47$	0.229
	TL	$F_{1,74} = 10.17$	**0.002**
SSI	Season	$F_{1,39} = 12.15$	**0.001**
n = 44	Sex	$F_{1,39} = 0.28$	0.601
$R^2 = 0.359$	Season × sex	$F_{1,39} = 0.01$	0.936
	TL	$F_{1,39} = 4.24$	**0.046**
BA (titer)	Season	$F_{1,83} = 4.69$	**0.033**
n = 88	Sex	$F_{1,83} = 6.53$	**0.012**
$R^2 = 0.246$	Season × sex	$F_{1,83} = 4.06$	**0.047**
	TL	$F_{1,83} = 8.52$	**0.005**
N:L	Season	$F_{1,80} = 0.15$	0.697
n = 85	Sex	$F_{1,80} = 1.06$	0.306
$R^2 = 0.000$	Season × sex	$F_{1,80} = 2.82$	0.097
	TL	$F_{1,80} = 0.04$	0.834
HCT (%)	Season	$F_{1,73} = 2.03$	0.158
n = 78	Sex	$F_{1,73} = 0.10$	0.750
$R^2 = 0.000$	Season × sex	$F_{1,73} = 0.57$	0.454

	TL	$F_{1,73} = 0.56$	0.458
CF	Season	$F_{1,90} = 0.26$	0.614
n = 95	Sex	$F_{1,90} = 0.57$	0.452
$R^2 = 0.066$	Season × sex	$F_{1,90} = 0.04$	0.846
	TL	$F_{1,90} = 6.44$	**0.013**

Abbreviations: Total body length (TL), estradiol (E2), androgens (A), gonadosomatic index (GSI), total white blood cell count (WBC), spleen-somatic index (SSI), bacterial agglutination (BA), neutrophil to lymphocyte ratio (N:L), hematocrit (HCT), condition factor (CF). coefficient of determination (R^2); sample size (n). Significant effects are depicted in bold.

Table 2. Linear models for immunological, reproductive and health-state parameters of adult sandperch (*Pinguipes brasilianus*). All models included season (reproductive or non-reproductive), sex (male or female) and their interaction as predictor variables. Total body length was included as a covariate.

Parameter	Predictor	*F*	*P*
E_2 (ng/ml)	Season	$F_{1,58} = 2.34$	0.132
n = 63	Sex	$F_{1,58} = 6.32$	**0.015**
$R^2 = 0.301$	Season × sex	$F_{1,58} = 5.88$	**0.018**
	TL	$F_{1,58} = 3.58$	0.064
A (ng/ml)	Season	$F_{1,58} = 2.13$	0.150
n = 63	Sex	$F_{1,58} = 0.94$	0.337
$R^2 = 0.060$	Season × sex	$F_{1,58} = 1.10$	0.299
	TL	$F_{1,58} = 0.09$	0.763
GSI	Season	$F_{1,62} = 32.10$	**<0.001**
n = 67	Sex	$F_{1,62} = 112.8$	**<0.001**
$R^2 = 0.747$	Season × sex	$F_{1,62} = 0.01$	0.919
	TL	$F_{1,62} = 0.01$	0.944

WBC (n)	Season	$F_{1,57} = 1.85$	0.179
$n = 62$	Sex	$F_{1,57} = 0.04$	0.836
$R^2 = 0.000$	Season × sex	$F_{1,57} = 0.63$	0.430
	TL	$F_{1,57} = 0.16$	0.687
SSI	Season	$F_{1,62} = 2.03$	0.159
$n = 67$	Sex	$F_{1,62} = 3.09$	0.084
$R^2 = 0.152$	Season × sex	$F_{1, 62} = 11.22$	**0.001**
	TL	$F_{1, 62} = 0.15$	0.704
BA (titer)	Season	$F_{1,54} = 9.63$	**0.003**
$n = 59$	Sex	$F_{1,54} = 0.57$	0.453
$R^2 = 0.216$	Season × sex	$F_{1,54} = 0.02$	0.877
	TL	$F_{1,54} = 2.39$	0.128
N:L	Season	$F_{1,58} = 5.59$	**0.021**
$n = 63$	Sex	$F_{1,58} = 1.50$	0.225
$R^2 = 0.160$	Season × sex	$F_{1,58} = 1.97$	0.166
	TL	$F_{1,58} = 0.01$	0.935
HCT (%)	Season	$F_{1,59} = 0.25$	0.616

$n = 64$	Sex	$F_{1,59} = 4.45$	**0.039**
$R^2 = 0.098$	Season × sex	$F_{1,59} = 0.16$	0.693
	TL	$F_{1,59} = 0.15$	0.703
CF	Season	$F_{1,61} = 0.03$	0.865
$n = 66$	Sex	$F_{1,61} = 0.56$	0.458
$R^2 = 0.000$	Season × sex	$F_{1,61} = 0.45$	0.505
	TL	$F_{1,61} = 0.32$	0.571

Abbreviations: Total body length (TL), estradiol (E2), androgens (A), gonadosomatic index (GSI), total white blood cell count (WBC), spleen-somatic index (SSI), bacterial agglutination (BA), neutrophils to lymphocytes ratio (N:L), hematocrit (HCT), condition factor (CF), coefficient of determination (R2); sample size (n). Significant effects are depicted in bold

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

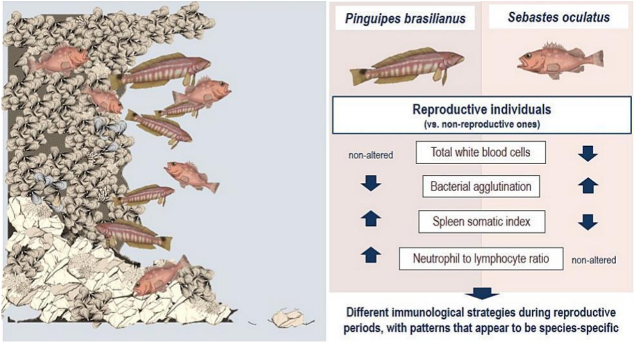
Graphical abstract

Highlights

1. The link between reproduction and immunity in two sympatric marine fish species was investigated.

2. Depending on fish species, total white blood cells, spleen index, natural antibodies and neutrophil to lymphocyte ratio differed between reproductive and non-reproductive individuals.

3. Our results suggest the existence of species-specific immunological strategies in reproductive versus non-reproductive periods and highlight the immune component-specificity of trade-offs between reproduction and immune function.



Pinguipes brasilianus	**Reproductive individuals** (vs. non-reproductive ones)	*Sebastes oculatus*
non-altered	Total white blood cells	⬇
⬇	Bacterial agglutination	⬆
⬆	Spleen somatic index	⬇
⬆	Neutrophil to lymphocyte ratio	non-altered

⬇

Different immunological strategies during reproductive periods, with patterns that appear to be species-specific

Graphics Abstract

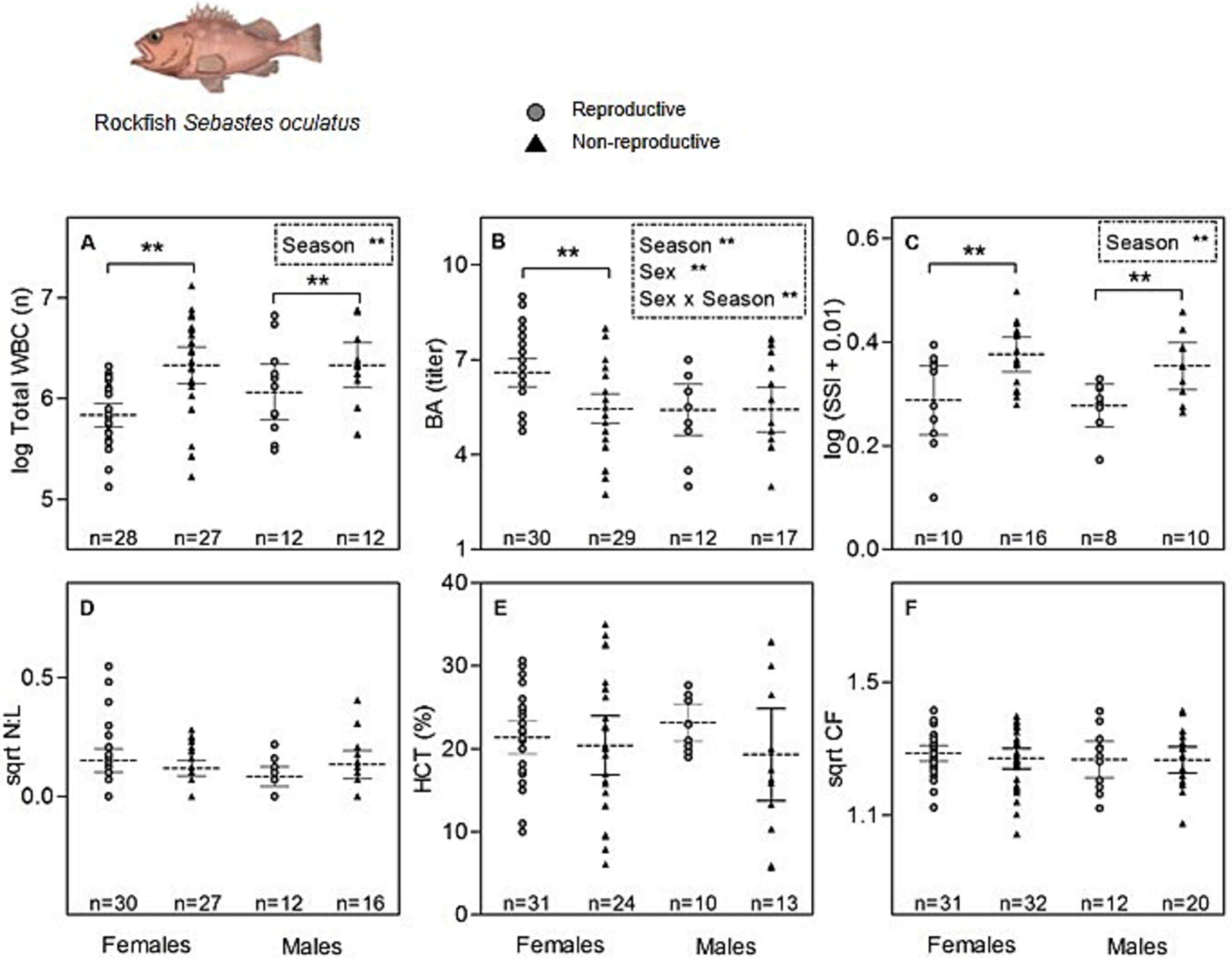

Rockfish *Sebastes oculatus*
Reproductive
Non-reproductive
A
Season **
**
**
log Total WBC (n)
5
6
7
n=28
n=27
n=12
n=12
B
Season **
Sex **
Sex x Season **
**
BA (titer)
1
4
7
10
n=30
n=29
n=12
n=17
C
Season **
**
**
log (SSI + 0.01)
0.0
0.2
0.4
0.6
n=10
n=16
n=8
n=10
D
sqrt N:L
0.0
0.5
n=30
n=27
n=12
n=16
E
HCT (%)
0
10
20
30
40
n=31
n=24
n=10
n=13
F
sqrt CF
1.1
1.5
n=31
n=32
n=12
n=20
Females
Males
Females
Males
Females
Males

Figure 1

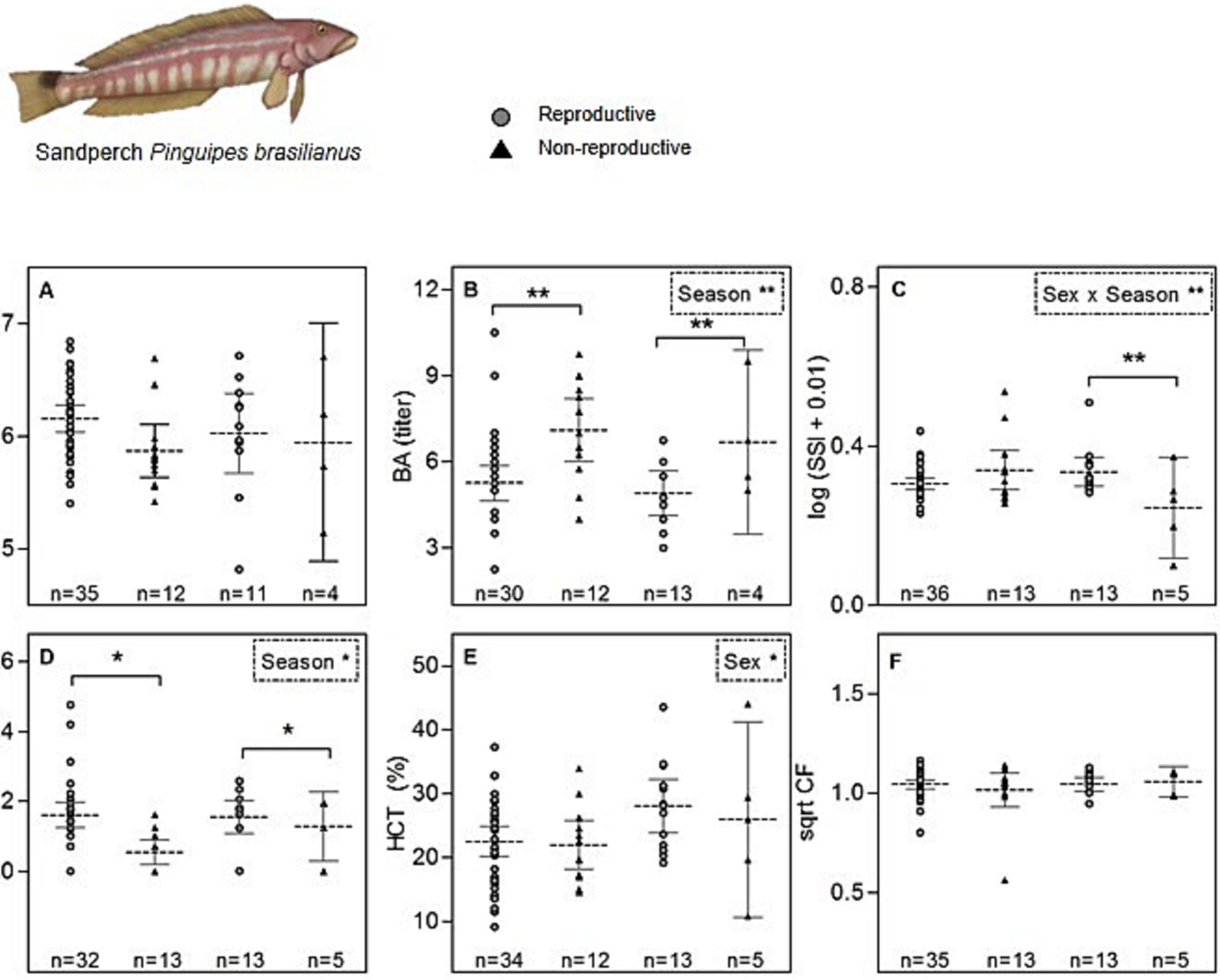

Sandperch *Pinguipes brasilianus*
Reproductive
Non-reproductive
A
log Total WBC (n)
7
6
5
n=35
n=12
n=11
n=4
B
Season **
**
**
BA (titer)
12
9
6
3
n=30
n=12
n=13
n=4
C
Sex x Season **
**
log (SSI + 0.01)
0.8
0.4
0.0
n=36
n=13
n=13
n=5
D
Season *
*
*
sqrt N:L ratio
0.6
0.4
0.2
0.0
n=32
n=13
n=13
n=5
E
Sex *
HCT (%)
50
40
30
20
10
n=34
n=12
n=13
n=5
F
sqrt CF
1.5
1.0
0.5
n=35
n=13
n=13
n=5
Females
Males
Females
Males
Females
Males

Figure 2

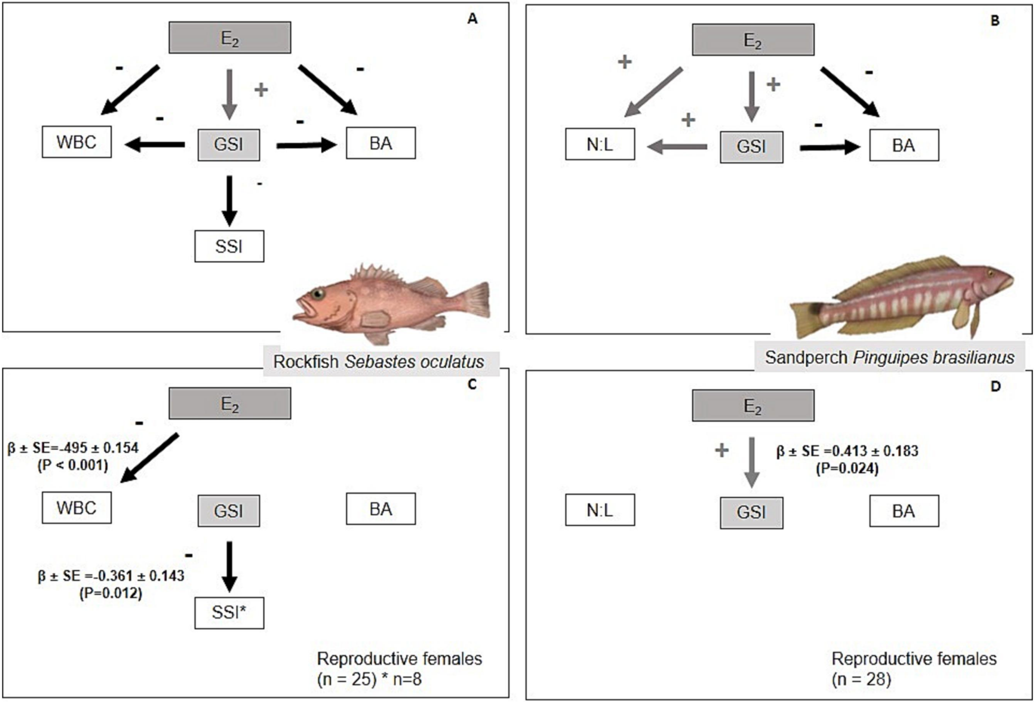

A
E2
-
+
-
WBC
-
GSI
-
BA
-
SSI
Rockfish Sebastes oculatus
B
E2
+
+
-
+
N:L
GSI
-
BA
Sandperch Pinguipes brasilianus
C
E2
-
β ± SE=-495 ± 0.154
(P < 0.001)
WBC
GSI
BA
-
β ± SE =-0.361 ± 0.143
(P=0.012)
SSI*
Reproductive females
(n = 25) * n=8
D
E2
+
β ± SE =0.413 ± 0.183
(P=0.024)
N:L
GSI
BA
Reproductive females
(n = 28)

Figure 3