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Journal:	*Evolution*
Manuscript ID	Draft
Manuscript Type:	Brief Communication
Keywords:	Behavior, Competition, Mating Systems, Reproductive Strategies, Selection - Sexual, cost of reproduction

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Abstract

Measuring the reproductive cost is crucial to understand of sexual conflict and its evolutionary outcomes. Sexual conflict is thought to originate from anisogamy – the size difference between male and female gametes; if sperm are tiny and not produced in vastly greater numbers than eggs, at any mating females' gametic investment is larger than that of males. Testing this prediction has proven difficult, especially because males and females differ in many more traits than just gamete size. We overcame this difficulty by exposing simultaneous hermaphrodite polychaete worms *Ophryotrocha diadema* (where the two sexual functions occur in the same body) to two social conditions, pairs and groups>2, where they invest either relatively more in the female function or more in the male function, respectively. Then we measured several markers of cellular oxidative status which is a physiological mediator of life-history strategies. Male-biased hermaphrodites produced fewer eggs but, unexpectedly, paid larger oxidative costs than female-biased hermaphrodites, which produced more eggs. Male-biased hermaphrodites compete for mating as males (hermaphrodites in pairs do not) suggesting that male competition might be costlier than egg production. This result suggests the need of including different reproductive traits, namely competition over egg fertilization, in the measures of reproductive costs.

Keywords: oxidative stress, cost of reproduction, sexual conflict, hermaphrodites

Introduction

The scientific literature on sexual selection and sexual conflict is largely based on the far-reaching consequences arising from anisogamy, the size difference between male and female gametes (Parker 1979, 2006; Leonard 2010; Schärer et al. 2012; Lehtonen et al. 2016). If both sexes allocate limited internal resources to reproduction, producing fewer large gametes rather than many more tiny ones implies that at any mating females spend more resources than males (Kokko & Jennions 2008). Although large variation exists, the resource investment in gametes is indeed disproportionately higher for females (up to 1000 times higher according to a meta-analysis, Hayward & Gillooly, 2011).

However, investment in gametes is only one component of reproductive costs, as other traits potentially contribute to the difference between the reproductive effort of the two sexes (Trivers 1972; sex role divergence, Kokko & Jennions 2008; Schärer et al. 2012). Males typically invest more than females on attempts to gain additional matings via mate search, mate competition, courtship, or territoriality (Queller, 1997). Females, on the other hand, often care for their offspring more than males (Kokko & Jennions 2008), although large variation exists among taxa (Trivers 1972; Gross, 2005). Even if the association between the different reproductive traits appears more complex than initially thought (e.g., Clutton-Brock 2007; Kokko & Jennions 2008), all of these behavioral and non-behavioral traits would require resources (e.g. nutrients, energy reserves, time) and contribute to the overall cost of reproduction - which, following Williams (2018), we define here as any negative effect of investment in current reproductive attempt on future fecundity and/or survival.

Identifying the differential cost that sexes pay to reproduction has consequences for our understanding of the evolutionary conflict between males and females, as well as in our comprehension of the evolution of life-history traits, where a negative trade-off is predicted between reproductive effort and lifespan (Stearns 1992). For instance, Trivers (1972) reasoned that, if both sexes care for offspring, females from iteroparous species are expected to pay more than males for current reproduction. However, a recent meta-analysis on the long-term cost of reproductive effort in birds found that males which cared for experimentally enlarged clutches (increased parental effort) had shorter lifespans than males of the control group, whereas their female partners did not, addressing sex-specific differences in reproductive costs (Santos & Nakagawa, 2012).

These results highlight that comparing reproductive costs between sexes is more difficult than expected. Indeed, the proximate mechanisms underpinning the cost of reproduction are unlikely to coincide in the two sexes. Males and females are morphologically dimorphic in many species, involving the development of costly morphological structures in only one sex (Heath 1977). In addition, reproductive investment includes costs related to sex-specific, dimorphic, physiological traits, such as sexual hormones, metabolism, immune responses, reproductive proteins, defenses against stress and toxicity (e.g., Harshman & Zera 2007). Finally, we lack evidence about whether males and females allocate similar resource budgets to reproduction (Kokko & Jennions 2008) and whether they adopt similar strategies in terms of resource allocation (Romero-Haro et al. 2016). For instance, females may invest all of their available reproductive resources into caring for current brood, whereas males may scale their investment on the perceived opportunities for reproductive success (Santos & Nakagawa 2012). In sum, comparing reproductive costs paid by males with those paid by females has proven challenging, because of the multitude of sex differences acting as confounding factors (e.g., Romero-Haro et al. 2016).

Hermaphrodites have both sexes tied together in the same body (Schärer & Ramm 2016) and offer the advantage of estimating the cost of male and female reproduction while minimizing confounding factors associated with sexes being expressed in different bodies, with sex-specific morphology, physiology, metabolism, or life-history traits. In addition, if it is true that simultaneous hermaphrodites (i.e., organisms that are males and females at the same time; hereafter, hermaphrodites) typically have both sexual functions active at any moment, they are able to vary their relative investment into the two sexual functions (plastic sex allocation, Charnov 1982; Schärer 2009; Koene 2017). They shift from a relatively more male-biased sex allocation (i.e., increased expression of male function) that they exhibit when they perceive multiple mating opportunities and sexually competitive conditions, to a relatively more female-biased sex allocation (i.e., increased expression of female function) under non-competitive conditions. Typically, when hermaphrodites are kept in pairs and their mating opportunity is limited to mating with the only available partner, they channel most of their reproductive resources to the female function (e.g., by investing more in ovary tissue, Janicke et al. 2013, or producing more eggs, Lorenzi et al. 2005), whereas they shift resources towards the male function when the number of potential mates/rivals increases and sexual selection becomes stronger (Lorenzi and Sella 2008). Although the trade-off between sexual functions is not always apparent in terms of female/male gametes or ovary/testis size (Schärer 2009), there is experimental evidence for plastic sex allocation (e.g., De Visser et al. 1994; Yund et al. 1997; Janicke et al. 2013), especially when the behavioral components of reproductive traits are included in the analyses (e.g., Santi et al. 2018; Picchi & Lorenzi 2019). Simultaneous hermaphrodites therefore prove to be convenient model organisms to measure the costs of male and female reproduction.

Here, we manipulated the sexually competitive environment of the simultaneously hermaphroditic *Ophryotrocha diadema* worms, which are small marine polychaetes, to get female-biased and male-biased individuals and then measured the cost of reproduction in terms of cellular oxidative stress in these two groups.

An accurate understanding of life-history trade-off requires knowledge of the underlying physiological costs of the biological functions involved (Flatt and Heyland 2013). There is increasing evidence for the role of cellular oxidative stress as one of these physiologic costs (Costantini 2014). Normal metabolism, including reproduction, produces reactive oxygen species (ROS) in all photosynthetic and aerobic organisms, which cause cellular damage (Brookes 2005). Organisms counteract ROS effects with protective antioxidant molecules, such as enzymes, vitamins and other molecules, which neutralize ROS, so that any decline in specific antioxidant or in integrative markers (e.g. antioxidant capacity of a given tissue) might reflect a compromisation of the antioxidant defenses. However, oxidation of biomolecules (e.g. thiols, fatty acids, DNA) is a continuous process that is unlikely to ever be exactly zero since ROS are continually produced and some oxidation is always generated (Costantini 2014). Oxidative stress has been addressed as a proximate cost of reproduction in both vertebrates and invertebrates and as one of the mechanisms underpinning the negative trade-off between reproduction/fecundity and longevity (Costantini 2008, 2014; Salmon et al. 2001; Alonso-Alvarez et al. 2004; Metcalfe & Monaghan 2013; Metcalfe & Alonzo-Alvarez 2010). Oxidative stress typically increases with reproductive effort, supporting its potential role in mediating a tradeoff between reproduction and lifespan (Blount et al. 2015; Georgiev et al. 2015). In vertebrates with no parental care, oxidative stress is typically higher in females than males (Costantini 2018) but evidence is conflicting. For instance, recently, Romero-Haro et al. (2016) found sex-specific, short-term responses to experimentally manipulated parental effort: males caring for enlarged clutches suffered higher oxidative damage than control males, whereas females did not.

In the current experiment we predicted that, if the behavioral and non-behavioral components of the female function are major costs of reproduction, worms with female-biased sex allocation – that is, with the stronger effort in the costlier sex function - would suffer higher oxidation of the antioxidant thiols and exhibit a lower antioxidant protection than worms with more male-biased sex allocation (stronger effort in the cheaper sex function). Therefore, we measured a panel of four oxidative status markers in worms kept in pairs (female-biased allocation and no competition) and in worms kept in groups (male-biased allocation and competition), controlling for density effects.

Material and methods

Ophryotrocha diadema is a 3-4 mm-long, simultaneously hermaphroditic polychaete worm living among the fouling fauna incrusting marine surfaces in harbors and likely living mainly at low – but fluctuating – population density, as typical for interstitial organisms (Westheide 1984).

When fully sexually mature, the worms produce many egg-clutches during their ~3-months-long reproductive life as they typically lay up to an egg-cocoon of about 25 eggs every 4 days (Picchi et al. 2018) (while also fertilizing those of their partners). They are obligate cross-fertilizers with external egg fertilization, and mate at any mating either as females or as males; the worm in the female role produces a jelly cocoon, releases eggs inside the cocoon, and the worm in the male role, in physical contact with the partner, releases its sperm inside the cocoon (pseudocopulation, Westheide 1984). In pairs, worms switch between sexual roles - from laying eggs to fertilizing partners' eggs - as often as every 2 days (Sella, 1985; Picchi et al. 2018). Sperm production is nearly continuous and they can fertilize partners' eggs multiple times in a row (Sella 1990), whereas the production of mature eggs occurs in bouts. Although costly for *O. diadema* small larva in terms of resource investment (larvae which fertilize more cocoons grow at a slower pace, Sella & Lorenzi 2003), producing sperm is likely less costly than producing eggs for mature hermaphrodites, at least in terms of volume of resources invested (Sella & Ramella 1999). Egg diameter is >60 times larger than sperm diameter (Pfannenstiel & Grünig 1990) and a single egg clutch requires up to 38% investment of bodily resources (Schleicherová et al. 2014). These hermaphrodites exhibit parental care (they alternate between sitting on their egg cocoons and moving back and forth on them possibly to free cocoons from fungi and bacteria with their mandibles, Sella 1991; XXX, pers. obs.) and do so significantly more if they laid the eggs (maternal care) than if they fertilized them (paternal care) (Picchi & Lorenzi 2019), adding time- and resource-consuming components to reproductive costs via the female function.

Overall, *O. diadema* investment in the female function appears to exceed that in the male function. Consistently, in the long term, the cumulative cost of the female function translates into diminished lifespan; in pairs, rare worms that reproduced only as males for extended time periods had significantly longer lifespan than worms that reproduced via both sexual functions (Di Bona et al. 2010). However, in general, worm longevity is higher in pairs than in groups (XXX, pers. obs.), possibly because worms in groups >2 overtly compete for fertilizing partners' eggs (Santi et al. 2018) suggesting that the cost of reproduction via the male function depends on the social context.

Rearing Worms

The worms used in the experiment originated from 230 pairs of parental worms. They were kept with their kin until they were newly fully sexually mature (with either immature eggs or ready-to-lay eggs, see below) and entered the experiment as same-age, virgin hermaphrodites (i.e., same past mating-history and future reproductive prospects). The worms were reared in artificial sea water in thermostatic cabinets at 21 °C and fed with spinach *ad libitum*.

Experimental Design

We aimed at testing oxidative status in female-biased and male-biased worms; that is, the two forms of sex allocation the worms exhibit when kept either in isolated pairs (2 worms) or in groups (>2 worms), respectively. To this aim, we randomly assigned 2783 worms to either pairs or groups (1258 worms to 629 pairs and 1525 worms to 305 groups – five worms per group), each pair or group hosted in a separate bowl. (These social conditions are biologically meaningful for hermaphrodites which live at low population density, Westheide 1984). Social conditions affect oxidative balance (Miyashita et al. 2006; Lardy et al. 2016). For this reason, pairs and groups were placed either in 10- or 20-mL water bowls (high or low density, respectively) to control for putative density effects on stress measures (e.g., effects of encounter rates, oxygen availability, metabolite/waste accumulation). Due to the large sample size, the experiment was split into three temporarily separated replicates (samples balanced by treatment and density among replicates) and included bowls with hermaphrodites with ready-to-lay eggs and bowls with hermaphrodites with immature eggs, as visually assessed by screening eggs through the worms' transparent body wall at the beginning of the experiment. The effect of egg maturity was included in the analyses to control for potential effects of resource budget on oxidative costs.

Following Schleicherová et al. (2006), the experiment lasted 9 days to allow worms to assess current mating opportunities and bias their sex allocation accordingly before measuring oxidative stress levels.

Measures of female investment

The worms' ability to adjust sex allocation as a function of perceived competitive condition (i.e., to shift resources from the male to the female function or vice versa depending on mate/rival availability, Charnov, 1982; Schärer 2009) is easily measured in this species in terms of egg and cocoon production, both measures being significantly higher for worms in pairs than in larger groups (Lorenzi 2005, 2006; Santi et al. 2018; Picchi & Lorenzi 2019). In contrast, previous experimental sperm counts showed that sperm production does not vary significantly with social condition (Lorenzi et al. 2005; 2006). Indeed, sperm are non-motile (aflagellate, Berruti et al. 1978) and released inside the egg cocoon (Westheide, 1984); sperm counts and sperm redundancy are very low (average sperm count of 1500 sperm per mature hermaphrodite, as measured via histological sections, and an estimate redundancy of 50 sperm per egg, Sella 1991). Therefore, we measured sex allocation as egg and cocoon production and counted the number of cocoons and eggs per cocoon produced in each bowl across the 9-days-experimental period.

Markers of oxidative status

On day 9, we collected the worms in Eppendorf vials (splitting them by treatment, density and egg maturity level; 47 vials in total, of which 25 worms from pairs and 22 worms from groups) and froze them at -20°C for the physiological analyses. We measured four markers of oxidative status in homogenates obtained from pools of ca. 50 individuals to get detectable concentrations. A decline in the concentrations of these particular markers is associated with cellular oxidative insults that compromise the antioxidant protection. We homogenised the worms in Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, France) supplemented with 1 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich, France) using a pestle. We measured the non-enzymatic antioxidant capacity using the OXY-Adsorbent test (Diacron International, Italy), which indicates the amount of HOCl neutralised by the non-enzymatic antioxidants that occur in the tissue; this marker was expressed as μmol HOCl neutralised/mg proteins; the concentration of thiols (a group of antioxidants) using the -SHp test (Diacron International, Grosseto, Italy) and expressed it as μmol/mg proteins; the activity of the antioxidant enzyme superoxide dismutase (SOD) using the Ransod assay (RANDOX Laboratories, France) and expressed it as units/mg proteins; and the activity of the antioxidant enzyme glutathione peroxidase (GPx) using the Ransel assay (RANDOX Laboratories, France) and expressed it as units/mg proteins. We ran all analyses in duplicate. We standardised the concentrations of markers by the amount of proteins (i.e. ratio between the antioxidant concentration and the protein concentration), as measured using the Bradford protein assay with albumin as reference standard (Sigma-Aldrich, France). We also measured the same markers in a sample of water contaminated with food remains and faeces to control for any interference on these measures (e.g., from bacteria or protozoans); values of absorbances quantified at the end of the reactions were not distinguishable by those of blanks, indicating a lack of interference.

Statistical analyses

We performed all statistical models using RStudio (version 1.1.463; R Core Team 2020; package lme4). We tested for the variation in female investment by performing Generalized Linear Mixed Effects Models (GLMM) on the number of eggs per cocoon (count data: Poisson distribution, log link) and included treatment (two levels: group, pair), density (high, low), level of egg maturity (immature eggs, ready-to-lay eggs) and experimental replicate (three levels) as predictors. Bowl ID and a case level random factors were entered in the model (the latter to account for overdispersion). We ran Linear Models (LM) to investigate for the source of variation of the average number of cocoons produced per individual during the experimental period and of the oxidative status markers (all variables were log transformed to meet the assumption of normal distribution of model residuals). As above, we tested for the effects of treatment while controlling for density, worm sexual maturity and experimental replicate.

We included the interactions treatment * density and treatment * level of egg maturity in all models. We removed non-significant interactions or factors only if this improved the fitting of the model by > 2 points as evaluated through the AIC value (Burnham & Anderson 2002).

We calculated the estimated marginal means of all variables in SPSS (version 23.0).

Results

In total, worms produced 3340 egg cocoons (1863 in pairs; 1477 in groups) and 58682 eggs (35843 in pairs; 22839 in groups). As expected, female investment differed significantly between social conditions. Worms produced on average more cocoons per individual and filled every cocoon with more eggs when they were in pairs than when they were in groups (Table 1, Fig 1). These results confirmed previous findings that worms invest significantly more in egg production when in pairs (female-biased sex allocation) than when kept in larger groups (which led to male-biased sex allocation) (Lorenzi et al. 2005, 2008; Santi et al. 2018).

Worms in pairs had significantly higher non-enzymatic antioxidant capacity, thiols, and SOD than worms in groups, where such capacity was lower (Table 2, Fig. 2). The activity of GPx did not differ significantly between treatments (Table 2, Figure 2), although the trend was consistent with the other markers. The differences in oxidative status markers between pairs and groups were consistent across density and egg maturity levels (as indicated by non-significant interactions, Table 2) and neither density nor egg maturity level explained the variation (i.e., they were non-significant terms) in any oxidative status marker.

Table 1. Outcomes of linear models testing for the source of variation in sex allocation. Each model also included a fixed factor experimental replicate to control for this source of variation. Reduced models are shown; non-significant interactions were retained if their removal did not improve the fitting of the model based on the AIC value. Significant P-values in bold.

Female investment measure	**Source of variation**	**Reference level**	**Level**	**Estimate**	**SE**	**χ^2 (GLMM); F (LM)**	**P**
Number of cocoons per individual (LM)	Treatment	Pair	Group	-0.023	0.014	2.696	0.101
	Density	High	Low	-0.004	0.009	0.162	0.687
	Egg maturity	Immature eggs	Ready-to-lay eggs	0.044	0.014	10.723	**0.001**
	Treatment*Density		Group*Low	0.010	0.016	0.377	0.540
	Treatment*Egg maturity		Group*Ready-to-lay eggs	-0.076	0.016	22.057	**<0.0001**
Number of eggs per cocoon (GLMM)	Treatment	Pair	Group	0.005	0.045	0.011	0.918
	Density	High	Low	-0.067	0.031	4.823	**0.028**
	Egg maturity	Immature eggs	Ready-to-lay eggs	0.185	0.042	19.341	**<0.0001**
	Treatment*Density		Group*Low	0.067	0.050	1.794	0.180
	Treatment*Egg maturity		Group*Ready-to-lay eggs	-0.297	0.051	32.917	**<0.0001**

Table 2. Outcomes of linear models testing for the source of variation in sex allocation and oxidative status markers. Each model also included a fixed factor experimental replicate to control for this source of variation (outcome not shown because biologically irrelevant). Non-significant interactions were retained if their removal did not improve the fitting of the model based on the AIC value. Significant P-values in bold.

Marker of oxidative status	**Source of variation**	**Reference level**	**Level**	**Estimate**	**SE**	**t**	**P**
OXY	**Treatment**	Group	Pair	0.158	0.068	2.322	**0.026**
	Density	Low	High	0.049	0.061	0.809	0.423
	Egg maturity	Immature eggs	Ready-to-lay eggs	0.133	0.083	1.601	0.118
	Treatment*Density			-0.038	0.084	-0.450	0.656
	Treatment*Egg maturity			-0.129	0.086	-0.108	0.140
Thiols	**Treatment**	Group	Pair	0.267	0.104	2.562	**0.014**
	Density	Low	High	0.016	0.094	0.166	0.869
	Egg maturity	Immature eggs	Ready-to-lay eggs	0.148	0.127	1.164	0.252
	Treatment*Density			-0.074	0.128	-0.575	0.569
	Treatment*Egg maturity			-0.210	0.131	-1.595	0.119
SOD	**Treatment**	Group	Pair	0.270	0.100	2.703	**0.010**
	Density	Low	High	0.106	0.090	1.179	0.246
	Egg maturity	Immature eggs	Ready-to-lay eggs	0.095	0.122	0.783	0.438
	Treatment*Density			-0.216	0.122	-1.764	0.086
	Treatment*Egg maturity			-0.159	0.126	-1.266	0.212
GPx	Treatment	Group	Pair	0.189	0.120	1.577	0.123
	Density	Low	High	0.027	0.107	0.251	0.803
	Egg maturity	Immature eggs	Ready-to-lay eggs	0.082	0.146	0.562	0.578
	Treatment*Density			-0.082	0.147	-0.557	0.581
	Treatment*Egg maturity			-0.093	0.151	-0.617	0.541

Table 3. Schematic summary of the relative investment (time and/or resources) in male and female reproductive traits in simultaneously hermaphroditic *O. diadema* worms: no investment is indicated as "-"; any investment is indicated as "+". When both sexual functions contribute to a trait, the function expressing the larger investment is indicated by "++". Worms in pairs invest more in the female function, whereas worms in groups invest more in the male function.

	Sex function involved		
Trait	female	male	References
Egg production	+	-	Schleicherová et al. 2014
Jelly cocoon production	+	-	Westheide 1984
Courtship	+	+	Lorenzi et al. 2018
Parental care (maternal or paternal care)	++	+	Picchi & Lorenzi 2019
Motility (mate search)	+	++	Picchi & Lorenzi 2019
Sperm production	-	+	Lorenzi et al. 2005
Mate competition (aggression)	-	+	Lorenzi et al. 2013; Santi et al. 2018

Discussion

It is increasingly recognized that the quantification of oxidative status markers provides a reliable physiological tool to estimate the metabolic/physiological cost of reproduction; oxidative stress is expected to increase with reproductive effort and is typically higher in females, which produce costly eggs, than in males (reviewed in Costantini 2018). Consequently, we expected that hermaphrodites in pairs, which produced 40% more eggs than hermaphrodites in groups, suffered higher oxidative stress. In contrast to our expectation, the hermaphrodites with the higher egg output (i.e., in pair) experienced lower oxidation of thiols and circulating non-enzymatic antioxidants and had higher levels of antioxidant enzymes compared to those with lower egg output and mating rates (i.e., in group). These results suggest that the cost of egg production is lower than male investment, indicating that gametic investment is only one component of the reproductive investment and that mating competition, which is often hard to quantify, is a prevalent cost in many reproductive systems.

Because worms in groups were more numerous and might have suffered a decline in antioxidant capacity because encounter rates were higher or metabolites accumulated in larger amounts, we controlled for density by keeping the number of worms constant and varying water volume per worm for both pairs and groups. The lack of significant effects of density suggests that neither encounter rates nor metabolite accumulation (or any other potential effect associated with density) accounted for the variation in oxidative status markers in our experimental conditions. Instead, being exposed to high or low sexually competitive condition (group or pair, respectively) did.

Although our model organism offers the advantage that male and female sex share the same body (e.g. same morphology, physiology), it has the limitation that individuals both produce eggs and fertilize partners' eggs. As a consequence, we cannot completely tease apart the cost they paid to reproduction as females or as males (i.e., the pure effect of variation in egg production cannot be calculated), yet, we can still examine their relative investment in reproductive traits other than egg production, such as courtship and sexual display, mating, mate competition, sperm/ejaculate expenditure, or parental care (summarized in Table 3).

The reproductive biology of these worms suggests that the investment in pre-mating behavioral competition is more relevant than sperm competition. Elaborate sexual traits are inherently costly, both if they consist of morphological traits (such as beetle horns or bird tails) or behavioral displays (Podos 2022). While morphological sexual traits have been rarely identified in hermaphrodites (but see love darts in land snails, Koene & Schulenburg 2005), some hermaphrodites spend a considerable amount of time in courtship, as reported for instance in reciprocating fish (Hart et al. 2016) and polychaete worms (Sella 1985). Courtship is costly in terms of resources (time and energy, Vehrencamp et al. 1989, but see Barske et al. 2014) as indirectly confirmed by birds economizing the amount of courtship under competitive conditions (Carmona-Isunza et al. 2015). *O. diadema* worms spend days in courting their partners - mostly by rubbing against each other - before mating (Lorenzi et al. 2018, 2021); however, courtship is highly symmetrical between partners irrespective of their perspective sexual role (Lorenzi et al. 2018) and does not vary significantly with competitive conditions (Lorenzi et al. 2006). This suggests that courtship is unlikely to explain the different physiological costs paid by male- *vs* female-biased worms.

Alternatively, acquiring mates and mating involve a cost. This has been recently measured in an elegant experimental test performed by Chung, Jennions and Fox (2021) on the eastern mosquitofish *Gambusia holbrooki,* a fish with internal fertilization. The authors showed that males who could see and olfactorily perceive females, but could not copulate because a mesh barrier separated them from females, payed lower costs to growth and immunocompetence than fish that could mate with females, even when males were experimentally prevented from ejaculating (via cutting the tip of the male gonopodium). Pure male mating effort is thus costly per se, even when ejaculation – and consequent sperm replenishment – does not take place, and even in the absence of mate competition (no rival male was present). We lack information on the cost of mating effort in hermaphrodites – both for mating as females and for mating as males. However, the number of cocoons per individual was higher in pairs than in groups – and each cocoon represents a mating (Westheide 1984). As a consequence, the higher physiological costs measured in groups cannot be ascribed to mating effort.

Sperm and ejaculate economics imply costs (Pitnick et al. 1995; Olsson et al. 1997; Sella & Lorenzi 2003), but we do not expect that the higher cost hermaphrodites paid in groups was associated to increased sperm expenditure. First, hermaphroditic *Ophryotrocha* worms typically have very low sperm counts (Sella 1991) and barely increase them in competitive conditions (Lorenzi et al. 2005, 2006) (suggesting that sperm allocation does not directly translates into gained matings). Second, although egg cocoons can be sired by multiple individuals (Lorenzi et al. 2014), sperm competition is likely limited since sperm are immotile (no tail, Morrow 2004). Finally, in isolated pairs of worms, those that reproduce mainly or exclusively as males have longer lifespan than partners that also reproduce as females (a typical female reproduction/lifespan trade-off, Di Bona et al. 2010), supporting the hypothesis that the pure male function is not that costly per se – at least if it only involves no more than producing sperm and mating.

Mate competition does not occur when worms live in pairs; in contrast, worms reared in larger groups compete with rivals for access to egg fertilization (Lorenzi et al. 2006; Santi et al. 2018). Typically, males respond to increased competition over egg fertilization by displaying behaviors that contribute to enhance their reproductive success (e.g. mate guarding, territorial defense and rival aggression; Birkhead & Møller 1998). *O. diadema* worms release sperm on freshly laid eggs inside the jelly cocoon; this requires the “male” to be in physical contact with the “female” by attaching to her tail so that he is driven inside the tubular egg-cocoon when the female exits it (Westheide 1984). Being in the right place (i.e., physical contact with the “female”) is therefore crucial to get access to egg fertilization. Evidence exists that mature hermaphrodites move and pursue each other, attack, injure and kill each other significantly more when they are in groups >2 worms - and that these behaviors are not associated with density and/or food competition but rather to competition over egg fertilization (Lorenzi et al. 2006; Santi et al. 2018; Picchi & Lorenzi 2019). In contrast, aggression is rare, if any, when worms are kept in pairs (Lorenzi et al. 2006; Santi et al. 2018), where they cooperate by reciprocating eggs (Picchi et al. 2018).

In conclusion, one explanation for the higher physiological cost paid by worms in groups would likely refer to competition for egg fertilization more than to any other trait (Table 3). In competitive conditions (groups), worms increase motility (Picchi & Lorenzi 2019) and exhibit overt attacks against rivals over the male role (Lorenzi et al. 2006; Santi et al. 2018). Mate competition has already been hypothesized as a predictor of sex differences in oxidative status in vertebrates. In a recent meta-analysis, females emerged as the sex which pays the higher oxidative costs only in species with no parental care, where the cost of reproduction is particularly high for females (because of egg production) (Costantini 2018). In all other species, male-male competition raised oxidative costs in males to levels similar to those paid by females (Costantini 2018).

Aggression implies direct costs - risk of injury and death - and opportunity costs, such as diminished opportunities for foraging or mate searching (Maynard Smith & Prize 1973; Frommen et al. 2019). It also implies enhanced metabolic activity and increased motility, whose cost might be apparent in terms of oxidative stress (Briffa & Sneddon 2007; Powers & Jackson 2008). In a natural population of the skink *Egernia whitii*, a lizard with large inter-individual variation in aggression in both males and females, the most aggressive males experienced higher oxidative stress (Isaksson et al. 2011). (This was not true for females (Isaksson et al. 2011), which highlights the limitations of comparing costs between sexes in separate-sex species and the advantage provided by using simultaneous hermaphrodites to this aim.). Similarly, among cichlid fish, *Pundamilia nyererei* males suffered higher oxidative stress levels than males of the less aggressive sibling species *P. pundamilia* during territorial contests (Dijkstra et al. 2011). In both examples, oxidative stress was associated to male mate-competition.

Competition with rivals over access to egg fertilization might occur at the expenses of the antioxidant protection in male-biased hermaphrodites as well. *O. diadema* hermaphrodites are able to quickly and plastically adjust their reproductive investment to current social conditions (Lorenzi 2005, 2006; Santi et al. 2018; Picchi & Lorenzi 2019), which suggests that population size fluctuates in the wild. However, many traits suggest they evolved as sparse population for extensive time periods. We lack information about the ecology and prevailing mating system of these almost microscopic, interstitial worms in the wild, but they exhibit many traits which are likely to have evolved under relaxed sexual selection, low sperm competition and a monogamous mating system. They have low sperm counts, non-motile sperm and in pairs they exhibit highly sophisticated reproductive tactics (conditional reciprocity, Picchi et al. 2018) and refined assessment of partner quality (Lorenzi et al. 2021).

In general, evidence is emerging that male pre-copulatory investment, including mate competition, entail large costs that have been overlooked in many gonochoristic and hermaphroditic species by the emphasis on post-copulatory sexual selection and the female perspective (for instance, in terms of senescence, review in Lemaître et al. 2015; Lemaître and Gaillard 2017). However, male ornaments in guppies (namely orange spots) have a higher senescence rate and a stronger impact on lifespan than post-copulatory traits (Gasparini et al. 2019). Similarly, mating effort trades off with body growth and immune function in eastern mosquitofish more than post-copulatory traits (*Gambusia holbrooki*, Chung et al. 2021).

Conclusions

The results of our experiment suggest that competition against rivals over egg fertilization results in larger oxidative costs than enhanced egg production in a simultaneous hermaphrodite. These results point out the relevance of including behavioral traits related to mate acquisition in the definition and quantification of reproductive costs (see also Santi et al. 2018; Picchi & Lorenzi 2019). In particular, for species whose reproductive traits seem specialized for reproducing under monogamy, pre-copulatory traits, and particularly mate competition, entails such high costs they overcome the cost of egg production.

References

Alonso-Alvarez C., Bertrand S., Devevey G., Prost J., Faivre B. and Sorci G. 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction. Ecol. Lett. 7: 363–368. doi:10.1111/j.1461-0248. 2004.00594.x

Barske J., Fusani L., Wikelski M., Feng N.Y., Santos M. and Schlinger B.A. 2014. Energetics of the acrobatic courtship in male golden-collared manakins (*Manacus vitellinus*). Proc. R. Soc. B 281: 20132482. doi: 10.1098/rspb.2013.2482

Berruti G., Ferraguti M., Lora Lamia Donin, C. 1978. The aflagellate spermatozoon of *Ophryotrocha*: a line of evolution of fertilization among polychaetes. *Gamete Res.* **1**, 287–292.

Birkhead T.R. and Møller A.P. 1998. Sperm competition and sexual selection. London, UK: Academic Press.

Blount J.D., Vitikainen E.I.K., Stott I. and Cant M.A. 2016. Oxidative shielding and the cost of reproduction. Biol. Rev. 91: 483–497. doi:10.1111/brv. 12179

Briffa M. and Sneddon L.U. 2007. Physiological constraints on contest behavior. Funct. Ecol. 21: 627–637.

Brookes P.S. 2005. Mitochondrial H+ leak and ROS generation: An odd couple. Free Radic. Biol. Med. 38: 12–23.

Burnham K.P., Anderson D.R. and Huyvaert K.P. 2011. AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. Behav. Ecol. Sociobiol. 65: 23–35. doi: 10.1007/s00265-010-1029-6

Carmona-Isunza M.C., Küpper C., Serrano-Meneses M.A. and Székely T. 2015. Courtship behavior differs between monogamous and polygamous plovers. Behav. Ecol. Sociobiol. 69: 2035–2042. doi: 10.1007/s00265-015-2014-x

Charnov E.L. 1982. The theory of sex allocation. Princeton, USA: Princeton University Press.

Chung M.-H.J., Jennions M.D. and Fox R.J. 2021. Quantifying the costs of pre- and post-copulatory traits for males: Evidence that costs of ejaculation are minor relative to mating effort. Evol. Lett. 5, 1–13. doi:10.1002/evl3.228

Clutton-Brock T. 2007. Sexual selection in males and females. Science 318: 1882–1885.

Costantini D. 2008. Oxidative stress in ecology and evolution: lessons from avian studies. Ecol. Lett. 11: 1238–1251. doi:10.1111/j.1461-0248.2008.01246.x

Costantini D. 2014. Oxidative stress and hormesis in evolutionary ecology and physiology. In: A marriage between mechanistic and evolutionary approaches. Berlin, Heidelberg: Springer-Verlag. pp. 1–362.

Costantini D. 2018. Meta-analysis reveals that reproductive strategies are associated with sexual differences in oxidative balance across vertebrates. Curr. Zool. 64: 1–11.

De Visser J.A.G.M., Ter Maat T., and Zonneveld C. 1994. Energy budgets and reproductive allocation in the simultaneous hermaphrodite pond snail, *Lymnaea stagnalis* (L.): a trade-off between male and female function. Am. Nat. 144: 861–867.

Di Bona V., Lorenzi M.C., Sella G. 2010. Functional males in pair-mating outcrossing hermaphrodites. Biol. J. Linn. Soc. 100: 451–456.

Dijkstra P.D., Wiegertjes G.F., Forlenza M., Van Der Sluijs I., Hofmann H.A., Metcalfe N.B. and Groothuis T.G.G. 2011. The role of physiology in the divergence of two incipient cichlid species. J. Evol. Biol. 24: 2639–2652.

Flatt T. and Heyland A. 2013. Mechanisms of life history evolution. The Genetics and Physiology of Life History Traits and Trade-Offs. Oxford, UK: Oxford University Press.

Frommen J.G. 2019. Aggressive communication in aquatic environments. Funct. Ecol. 34: 364–380.

Gasparini C., Devigili A. and Pilastro A. 2019. Sexual selection and ageing: Interplay between pre- and post-copulatory traits senescence in the guppy. Proc. Roy. Soc. B: 286: 1–9. doi: 0.1098/rspb.2018.2873

Georgiev A.V., Thompson M.E., Mandalaywala T.M., Maestripieri D. 2015. Oxidative stress as an indicator of the costs of reproduction among free-ranging rhesus macaques. J. Exp. Biol. 218: 1981-1985.

Gross M.R. 2005. The evolution of parental care. Q. Rev. Biol. 80: 37–45.

Harshman L.G. and Zera A.J. 2007 The cost of reproduction: the devil in the details. Trends Ecol. Evol. 22: 80–86. doi: 10.1016/j.tree.2006.10.008

Hart M.K., Kratter A.W. and Crowley P.H. 2016. Partner fidelity and reciprocal investments in the mating system of a simultaneous hermaphrodite. Behav. Ecol. 27: 1471–1479.

Hayward A. and Gillooly J.F. 2011. The cost of sex: quantifying energetic investment in gamete production by males and females. PLoS ONE 6: e16557.

Heath D.J. 1977. Simultaneous hermaphroditism: cost and benefit. J. Theor. Biol. 64: 363–373.

Isaksson C., While G.M., Mcevoy J., van de Crommenacker J., Olsson M., Groothuis T.G.G., Komdeur J. and Wapstra E. 2011. Aggression, but not testosterone, is associated to oxidative status in a free-living vertebrate. Behaviour 148: 713–731.

Janicke T., Marie-Orleach L., De Mulder K., Berezikov E., Ladurner P., Vizoso D.B. and Schärer L. 2013. Sex allocation adjustment to mating group size in a simultaneous hermaphrodite. Evolution. 67: 3233–3242.

Kokko H. and Jennions M.D. 2008. Parental investment, sexual selection and sex ratios. J. Evol. Biol. 21: 919–948.

Koene J.M. and Schulenburg H. 2005. Shooting darts: Co-evolution and counter-adaptation in hermaphroditic snails. BMC Evol. Biol. 5: 25.

Koene J.M. 2017. Sex determination and gender expression: Reproductive investment in snails. Mol. Reprod. Dev. 84: 132–143.

Lardy S., Rey B., Salin K., Voituron Y. and Cohas A. 2016. Beneficial effects of group size on oxidative balance in a wild cooperative breeder. Behav. Ecol. 27: 1820–1825.

Lehtonen J., Parker G.A. and Schärer L. 2016. Why anisogamy drives ancestral sex roles. Evolution 70: 1129–1135. doi:10.1111/evo.12926

Lemaître J.-F., Berger V., Bonenfant C., Douhard M., Gamelon M., Plard F., Gaillard J.-M. 2015. Early-late life trade-offs and the evolution of ageing in the wild. Proc. Roy. Soc. B 282: 20150209. http://dx.doi.org/10.1098/rspb.2015.0209

Lemaître J. F. and Gaillard J.M. 2017. Reproductive senescence: new perspectives in the wild. Biol. Rev. 92: 2182–2199.

Leonard J.L. 2010. The evolution of sexes, anisogamy, and sexual systems. Natural versus sexual selection. In J. L. Leonard and A. Cordoba-Aguilar, eds. The Evolution of primary sexual characters in animals. New York: Oxford Univ. Press. pp. 15–39.

Lorenzi M.C. and Sella G. 2008. A measure of sexual selection in hermaphroditic animals: parentage skew and the opportunity for selection. J. Evol. Biol. 21: 827–833. doi: 10.1093/beheco/11.3.260

Lorenzi M.C., Schleicherovà D. and Sella G. 2008. Sex adjustments are not functionally costly in simultaneous hermaphrodites. Mar. Biol. 153: 599–604.

Lorenzi M.C., Schleicherovà, D. and Sella G. 2014. Multiple paternity and mate competition in non-selfing, monogamous, egg-trading hermaphrodites. Acta Ethol. 17: 173–179. doi: 10.1007/s10211-013-0169-x

Lorenzi M. C., Araguas A., Bocquet C., Picchi L. & Ricci-Bonot C. 2018. Courtship behavior as a war of attrition in a simultaneous hermaphrodite. Anim. Biol. 69: 47–62.

Lorenzi M.C., Schleicherová D. & Sella G. 2006. Life history and sex allocation in the simultaneously hermaphroditic polychaete worm *Ophryotrocha diadema*: The role of sperm competition. Integr. Comp. Biol. 46: 381–389.

Lorenzi M.C., Schleicherovà D., Robles-Guerrero F.G. and Dumas M. 2021. Egg trading worms start reciprocation with caution, respond with confidence and care about partners' quality. Scientific Reports 11: 10552. doi: 10.1038/s41598-021-89979-7

Lorenzi M.C., Sella G., Schleicherová D. and Ramella L. 2005. Outcrossing hermaphroditic polychaete worms adjust their sex allocation to social conditions. J. Evol. Biol. 18: 1341–1347.

Maynard Smith J. and Price G. R. 1973. The logic of animal conflicts. Nature 246: 15–18.

Metcalfe N.B. and Monaghan P. 2013. Does reproduction cause oxidative stress? An open question. Trends Ecol. Evol. 28, 347–350. doi: 10.1016/j.tree.2013. 01.015.

Metcalfe N.B. and Alonso-Alvarez C. 2010. Oxidative stress as a life- history constraint: The role of reactive oxygen species (ROS) in shaping phenotypes from conception to death. Funct. Ecol. 24: 984–996.

Miyashita T., Yamaguchi T., Motoyama K., Unno K., Nakano Y., Shimoi K. 2006. Social stress increases biopyrrins, oxidative metabolites of bilirubin, in mouse urine. Biochem. Biophys. Res. Commun. 349: 775–780.

Morrow E.H. 2004. How the sperm lost its tail: the evolution of aflagellate sperm. Biol. Rev. Camb. Philos. Soc. 79: 795–814.

Olsson M., Madsen T. and Shine R. 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. Proc. Roy. Soc. B. 264: 455–459.

Parker G.A. 1979. Sexual selection and sexual conflict. In: Sexual selection and reproductive competition in insects (eds M. S. Blum, N. A. Blum), pp. 123–166. New York, NY: Academic Press.

Parker G.A. 2006. Sexual conflict over mating and fertilization: An overview. Phil. Trans. Roy. Soc. B 361: 235–259.

Pfannenstiel H.D. and Grünig C. 1990. Spermatogenesis and sperm ultrastructure in the polychaete genus *Ophryotrocha* (Dorvilleidae). Helgoländer Meeresun 44: 159–171.

Picchi L. and Lorenzi M.C. 2019. Gender-related behaviors: evidence for a trade-off between sexual functions in a hermaphrodite. Behav. Ecol. 30: 770–784. doi.org/10.1093/beheco/arz014

Picchi L., Cabanes G., Ricci-Bonot C. and Lorenzi M. C. 2018. Quantitative matching of clutch size in reciprocating hermaphroditic worms. Curr. Biol. 28: 3254–3259.

Pitnick S., Markow T.A. and Spicer G.S. 1995. Delayed male maturity is a cost of producing large sperm in Drosophila. Proc. Natl. Acad. Sci. U.S.A. 92: 10614–10618.

Podos J. 2022. Costs, constraints and sexual trait elaboration. Anim. Behav. 184: 209–214. doi: 10.1016/j.anbehav.2021.05.021

Powers S.K. and Jackson M. J. 2008. Exercise-induced oxidative stress: Cellular mechanisms and impact on muscle force production. Physiol. Rev. 88: 1243–1276. doi:10.1152/physrev.00031.2007.

Queller D.C. 1997. Why do females care more than males? Proc. R. Soc. Lond. B 264: 1555–1557.

Romero-Haro A.A., Sorci G., Alonso-Alvarez C. 2016 The oxidative cost of reproduction depends on early development oxidative stress and sex in a bird species. Proc. Roy. Soc. B 283: 20160842. doi: 10.1098/rspb.2016.0842

Salmon A.B., Marx D.B. and Harshman L.G. 2001. A cost of reproduction in *Drosophila melanogaster*: stress susceptibility. Evolution 55: 1600–1608. doi: 10.1111/j.0014-3820.2001.tb00679.x

Santi M., Picchi L. and Lorenzi M.C. 2018. Dynamic modulation of reproductive strategies in a simultaneous hermaphrodite and the preference for the male role. Anim. Behav. 146: 87–96. doi: 10.1016/j.anbehav.2018.10.004

Santos E.S. and Nakagawa S. 2012. The costs of parental care: a meta-analysis of the trade-off between parental effort and survival in birds. J. Evol. Biol. 25: 1911–1917. doi: 10.1111/j.1420-9101.2012.02569.x

Schärer L. 2009. Tests of sex allocation theory in simultaneously hermaphroditic animals. Evolution 63: 1377–1405.

Schärer L. and Ramm S.A. 2016. Hermaphrodites. In: Kliman, R. (ed.). Encyclopedia of Evolutionary Biology. Elsevier. Vol. 2. pp. 212-224.

Schärer L., Rowe L. and Arnqvist G. 2012. Anisogamy, chance and the evolution of sex roles. Trends Ecol. Evol. 27: 260–264. doi: 10.1016/j.tree.2011.12.006

Schleicherová D., Lorenzi M.C. and Sella G. 2006. How outcrossing hermaphrodites sense the presence of conspecifics and suppress female allocation. Behav. Ecol. 17: 1-5. doi: 10.1093/beheco/ari093

Schleicherová D., Sella G., Meconcelli S., Simonini R., Martino M. P., Cervella P., Lorenzi M.C. 2014. Does the cost of a function affect its degree of plasticity? A test on plastic sex allocation in three closely related species of hermaphrodites. J. Exp. Mar. Biol. Ecol. 453: 148–153. doi: 10.1016/j.jembe.2014.01.010

Sella G. 1990. Sex allocation in the simultaneously hermaphroditic polychaete worm *Ophryotrocha diadema*. Ecology 71: 27–32.

Sella G. 1991. Evolution of biparental care in the hermaphroditic polychaete worm *Ophryotrocha diadema*. Evolution 45: 63–68.

Sella G. 1985. Reciprocal egg trading and brood care in a hermaphroditic polychaete worm. Anim. Behav. 33: 938–944.

Sella G. and Lorenzi M.C. 2003. Increased sperm allocation delays body growth in a protandrous simultaneous hermaphrodite. Biol. J. Linn. Soc. 78: 149–154.

Sella G. and Ramella L. 1999. Sexual conflict and mating systems in the dorvilleid genus *Ophryotrocha* and the dinophilid genus *Dinophilus*. Hydrobiologia 402: 203–213.

Stearns S.C. 1992. The evolution of life histories. Oxford University Press.

Trivers R.L. 1972. Parental investment and sexual selection. In: Sexual selection and the descent of man, 1871–1971 (ed. B Campbell), Chicago, IL: Aldine. pp. 136–179.

Vehrencamp S.L., Bradbury J.W. and Gibson R.M. 1989. The energetic cost of display in male sage grouse. *Anim. Behav.* 38: 885–896. doi:10.1016/S0003-3472(89)80120-4.

Westheide W. 1984. The concept of reproduction in polychaetes with small body size: adaptations in interstitial species. Fortschr. Zool. 29: 265–287.

Williams T.D. 2018. Physiology, activity and costs of parental care in birds. J. Exp. Biol. 221: jeb169433. doi:10.1242/jeb.169433

Yund P. O., Marcum Y. and Stewart-Savage J. 1997. Life-history variation in a colonial ascidian: broad-sense heritabilities and tradeoffs in allocation to asexual growth and male and female reproduction. Biol. Bull. 192: 290–299.

Legend to the figures

Figure 1. Female allocation. Worms in pairs exhibited a relatively more female-biased sex-allocation than those in groups; they produced significantly more cocoons per individual (A), and their cocoons contained significantly more eggs (B). Hermaphrodites that entered the experiment with immature eggs had lower fertility (fewer cocoons and eggs per cocoon) than those with ready-to-lay eggs and were less efficient in adjusting their sex allocation to social condition. Figures show estimated marginal means (± s.e.).

Figure 2. Oxidative status markers. Worms in groups exhibited a higher depletion of oxidative status markers than those in pairs: the difference was significant for the non-enzymatic OXY marker (A), for the Thiols molecule (B) and for one of the two enzymatic markers SOD (C), while it was not for the GPx (D), although the tendency is consistent.
Figures show estimated marginal means (± s.e.).

639 Figure 1.

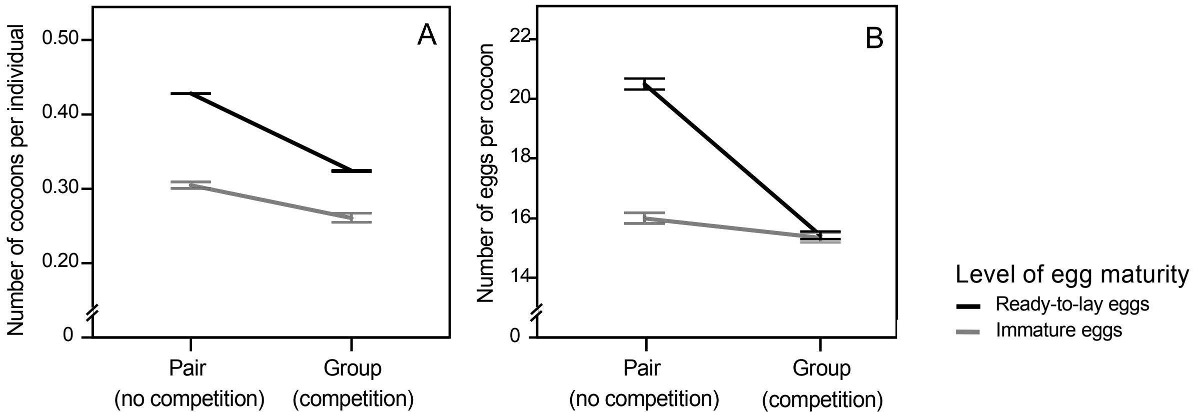

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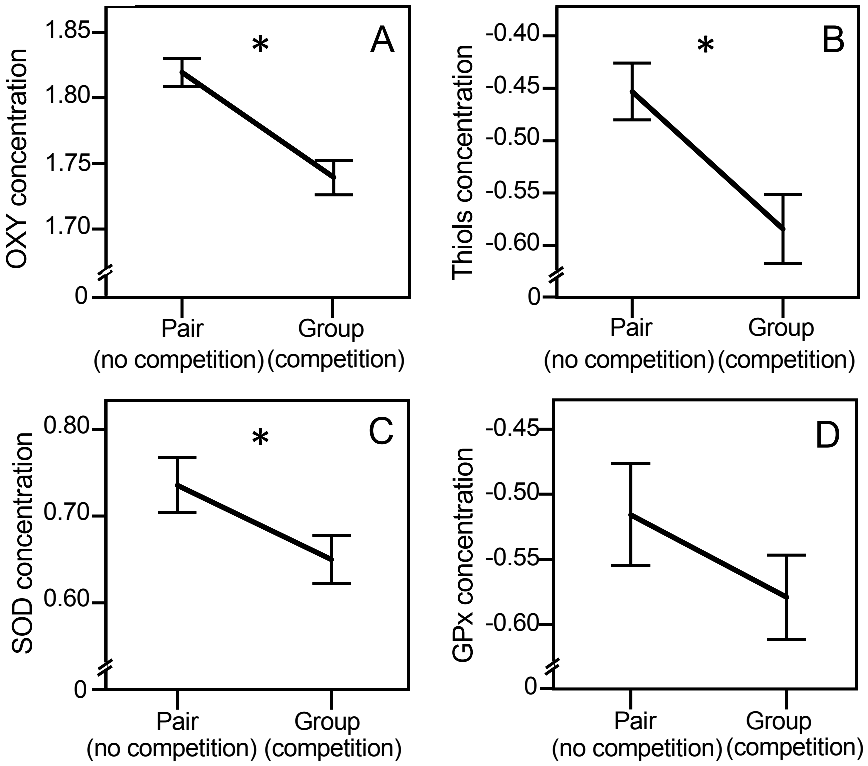
Figure 2


A
*
OXY concentration
1.85
1.80
1.75
1.70
0
B
*
Thiols concentration
-0.40
-0.45
-0.50
-0.55
-0.60
0
C
*
SOD concentration
0.80
0.70
0.60
0
D
GPx concentration
-0.45
-0.50
-0.55
-0.60
0
Pair
(no competition)
Group
(competition)