The effects of sex hormones on immune function: a meta-analysis

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ABSTRACT

The effects of sex hormones on immune function have received much attention, especially following the proposal of the immunocompetence handicap hypothesis. Many studies, both experimental and correlational, have been conducted to test the relationship between immune function and the sex hormones testosterone in males and oestrogen in females. However, the results are mixed. We conducted four cross-species meta-analyses to investigate the relationship between sex hormones and immune function: (1) the effect of testosterone manipulation on immune function in males, (2) the correlation between circulating testosterone level and immune function in males, (3) the effect of oestrogen manipulation on immune function in females, and (4) the correlation between circulating oestrogen level and immune function in females. The results from the experimental studies showed that testosterone had a medium-sized immunosuppressive effect on immune function. The effect of oestrogen, on the other hand, depended on the immune measure used. Oestrogen suppressed cell-mediated immune function while reducing parasite loads. The overall correlation (meta-analytic relationship) between circulating sex hormone level and immune function was not statistically significant for either testosterone or oestrogen despite the power of meta-analysis. These results suggest that correlational studies have limited value for testing the effects of sex hormones on immune function. We found little evidence of publication bias in the four data sets using indirect tests. There was a weak and positive relationship between year of publication and effect size for experimental studies of testosterone that became non-significant after we controlled for castration and immune measure, suggesting that the temporal trend was due to changes in these moderators over time. Graphical analyses suggest that the temporal trend was due to an increased use of cytokine measures across time. We found substantial heterogeneity in effect sizes, except in correlational studies of testosterone,

even after we accounted for the relevant random and fixed factors. In conclusion, our results provide good evidence that testosterone suppresses immune function and that the effect of oestrogen varies depending on the immune measure used.

Key words: honest signals, immune function, immunocompetence handicap hypothesis, meta-analysis, oestrogen, secondary sexual traits, sex hormones, sexual selection, testosterone, trade-offs.

CONTENTS

I. INTRODUCTION

There has been a long-standing interest in the effects of sex hormones on immune function (Ansar Ahmed, Penhale & Talal, 1985; Grossman, 1985; Schuurs & Verheul, 1990; Klein, 2004; Bouman, Heineman & Faas, 2005). Across species, females typically show stronger immune responses to parasitic challenges compared to males (Klein, 2004; Zuk & McKean, 1996). The prevalence and intensity of parasite infections also tend to be lower in females (Klein, 2004; Schuurs & Verheul, 1990). These sex differences were found even when experiments control carefully for parasite exposure (Daniels & Belosevic, 1994; Klein, Gamble & Nelson, 1999). These results suggest that sex differences in immune function are at least partly caused by physiological differences between the sexes. Among the physiological factors that differ between the sexes, sex hormones appear to be prime candidates as factors affecting immune function. The presence of testosterone and oestrogen receptors on various immune organs and immune cells suggests that sex hormones can influence the immune system directly (Alexander & Stimson, 1988; Cutolo *et al*., 1996; Danel *et al*., 1983; Roberts, Walker & Alexander, 2001; Wunderlich *et al*., 2002). Furthermore, the removal of gonads, one of the main sources of sex hormones, can alter immune functioning (e.g. Kamis, Ahmad & Badrul-Munir, 1992; Rivero *et al*., 2002; also see review by Klein, 2004).

Much attention has been placed on the effects of sex hormones, especially following the proposal of the influential immunocompetence handicap hypothesis (ICHH; Folstad & Karter, 1992). According to indirect-benefit models of female mate choice, females can obtain genetic benefits for their offspring by favouring the most ornamented males (Fisher, 1958; Pomiankowski, 1988). However, if females are consistent in their preference, genetic variance in fitness-related traits should be lost, leading to the so-called 'lek paradox' (Borgia, 1979; Taylor & Williams, 1982; Kirkpatrick & Ryan, 1991). Hamilton & Zuk (1982) proposed that male sexual signals might reflect genes that code for superior parasite resistance. Based on this hypothesis, male genetic variation is maintained through a co-evolutionary arms race where genes that code for good health spread among host individuals while parasites evolve increased virulence in response to the improved immunity of hosts. In elaboration of the Hamilton–Zuk hypothesis, the ICHH suggested that sex hormones, in particular testosterone in males, provide the mechanistic link between sexual signals and genes that code for good health through their effects on both signal development and immune function.

Since its inception, there has been much debate concerning the ICHH, particularly the assumption that sex hormones affect immune function. Roberts, Buchanan & Evans (2004) published a meta-analysis examining the effect of testosterone on immune function using studies from evolutionary biology. They found little support for the hypothesis that testosterone suppresses immune function in males. In the decade since then, many more studies on the effects of testosterone have been published, and meta-analytic techniques have advanced considerably. Therefore, we provide an update on Roberts *et al*. (2004). In addition, we examine the

132 relationship between oestrogen and immune function in females, which has not been
133 subjected to meta-analysis previously.
134
135 **(1) The effect of testosterone on immune function in males**
136 Males, in general, face intense mating competition (Trivers, 1972). As a result,
137 males from many species often develop elaborate testosterone-dependent secondary
138 sexual ornaments for the purpose of fighting for and attracting females (Andersson,
139 1994). The development of such ornaments is not without costs. Resources (e.g.
140 energy) are limited. Therefore, natural selection is expected to favour an optimum
141 allocation of energetic resources depending on the environmental situation, leading to
142 trade-offs between different fitness components (Stearns, 1977, 1992). According to
143 the ICHH, male sexual ornaments provide honest signals of the males' immune
144 function due to the trade-off between ornament development and immune health *via*
145 the effects of testosterone. Ornament development triggers a down-regulation of
146 immune function, which is effected through the immunosuppressive effect of
147 testosterone. This immunosuppression makes it impossible for males of low genetic
148 quality to develop exaggerated ornaments without compromising their health and
149 potential future reproductive success. Thus, only high-genetic-quality males can
150 afford to sustain the high levels of testosterone required for the development of
151 elaborate ornaments.
152 Many empirical studies have therefore examined the relationship between sex
153 hormones and immune function *in vivo*, across a wide variety of species and using
154 both experimental and correlational designs. For testosterone, both positive (e.g.
155 Bilbo & Nelson, 2001; Evans, Goldsmith & Norris, 2000; Morales-Montor *et al*.,
156 2002) and negative (e.g. Alonso-Alvarez *et al.*, 2007*a*; Belliure, Smith & Sorci, 2004;

157 Duckworth, Mendonça & Hill, 2001; Duffy *et al*., 2000) effects have been reported in
158 experimental studies.

159 Similarly, both positive and negative correlations between testosterone and
160 immune function have been reported (e.g. Duffy & Ball, 2002; Peters, 2000; Rantala
161 *et al*., 2012). In an immunocompetence handicap, individuals of different genetic
162 quality are expected to have different optimal hormone levels due to differences in the
163 marginal fitness benefit for every unit increase in a sexual signal (Getty, 2006).
164 Hence, both positive and negative correlations between testosterone and immune
165 function can be taken as supportive of an immunosuppressive effect of testosterone
166 (Getty, 2006). A positive correlation might suggest that individuals with high genetic
167 quality are able to cope with the reduced immune function that results from their high
168 levels of testosterone. By contrast, a negative correlation might suggest that healthy
169 individuals are trading their survival advantage for increased mating success.
170

171 **(2) The effect of oestrogen on immune function in females**

172 In females, trade-offs occur between the allocation of resources to current
173 reproduction and conserving resources for future reproduction (Stearns, 1992;
174 Thornhill & Gangestad, 2008). Oestrogen is critically involved in a number of female
175 reproductive functions, such as fertility and pregnancy (Ellison, 2001). Oestrogen is
176 also implicated in the production of sexual signals in species such as humans (*Homo*
177 *sapiens*) (Jasieńska *et al*., 2004; Moore *et al*., 2011; Smith *et al*., 2006) and red-sided
178 garter snakes (*Thamnophis sirtalis parietalis*) (Parker & Mason, 2012). Therefore, the
179 effect of oestrogen on immune function might be linked to trade-offs involving these
180 reproductive functions. However, the predicted direction of the effect of oestrogen on
181 immune function is unclear. Although an immune-enhancing effect seems intuitive

given that females tend to have better immune function (i.e. lower parasitism and stronger immune responses) than males, we should note that females can have better immune function than males even if oestrogen is immunosuppressive. As long as testosterone suppresses immune function in males and the immunosuppressive effect is stronger than that of oestrogen in females, we will see better immune function in females.

Similar to the results of studies looking at the effect of testosterone on immune function in males, the results of *in vivo* studies looking at the relationship between oestrogen and immune function in females have been mixed. Both positive (e.g. Ádori *et al*., 2010; Ding & Zhu, 2008; Zhou *et al*., 2011) and negative (e.g. Douin-Echinard *et al*., 2011; Salem *et al*., 2000) effects have been reported for experimental studies. Some researchers have observed that the effect of oestrogen appears to depend on the immune component measured and the oestrogen dosage used (Cutolo *et al*., 1996; Klein, 2004). Both positive and negative correlations between oestrogen level and immune function have also been reported (e.g. Klein & Nelson, 1997; Vainikka *et al*., 2004).

(4) This study

The mixed results in the literature make it difficult to assess the general effects of sex hormones on immune function. In this study, we use meta-analysis to analyse the results quantitatively from the literature. We provide an update on Roberts *et al*. (2004) by including studies conducted since then. We also analyse correlations between testosterone and immune function, which has not been done before. In addition, we examine the effect of oestrogen on immune function and the correlation between circulating oestrogen level and immune function in females by quantitatively

207 analysing the results from experimental and correlational studies, respectively, neither
208 of which has been done previously. Notably, for all these analyses, we include studies
209 from fields other than evolutionary biology, such as the biomedical sciences.
210 We conduct four phylogenetic meta-analyses (Verdú & Traveset, 2005;
211 Hadfield & Nakagawa, 2010; Nakagawa & Santos, 2012) to address the following
212 questions: (1) does experimental manipulation of testosterone affect immune function
213 in males? (2) Are the levels of circulating testosterone correlated with immune
214 function in males? (3) Does experimental manipulation of oestrogen affect immune
215 function in females? (4) Are the levels of circulating oestrogen correlated with
216 immune function in females?
217 We also conduct moderator analyses to investigate the factors that account for
218 variation in effect sizes. First, we look at sample-related variables such as the mating
219 system of the species and whether the individuals were sampled from natural or
220 laboratory populations. Polygamous species face more intense mating competition
221 compared to monogamous species (Andersson, 1994; Darwin, 1871). They tend to
222 have higher sex hormone levels and rely more on sex-hormone-mediated traits for
223 mating success (Andersson, 1994). Therefore, we expect the effect of sex hormones
224 on immune function to be stronger in polygamous species.
225 We also look at natural *versus* laboratory populations. Some laboratory
226 populations might have undergone artificial selection for traits that make the
227 populations ideal for laboratory experiments, including traits that might be related to
228 sex hormones, such as aggression. Therefore the results from laboratory populations
229 might be different to those from natural populations.
230 Second, we look at immune-measure-related variables such as the immune
231 measure used and whether the study measured baseline immunity or immune

232 reactivity to a pathogenic challenge. Different immune measures can be independent
233 from each other (Adamo, 2004). Furthermore, the effect of oestrogen appears to
234 depend on the immune component measured (Cutolo *et al*., 1996; Klein, 2004).
235 Therefore, we include immune measure as a moderator variable.

236 It has been suggested that measures of immune reactivity to pathogenic
237 challenges are more valid measures of immune function than baseline measures
238 because higher baseline immunity may indicate current infection status rather than
239 actual immunocompetence (Demas *et al*., 2011; Norris & Evans, 2000). We therefore
240 investigate whether effect sizes are different for baseline immunity *versus* immune
241 reactivity.

242 Third, for experimental studies, we look at the dosage of the hormones used
243 (physiological *versus* supraphysiological dosages) to ascertain whether the effects of
244 sex hormones are biologically relevant or simply due to overdosing. We also look at
245 whether steps were taken to control for endogenous production of sex hormones (i.e.
246 castration for males and ovariectomy for females).

247 Finally, to validate the robustness of our results, we present results from
248 analyses that provide indirect estimates of publication bias.
249

250 **II. METHODS**

251 **(1) Literature search**

252 We conducted a literature search between January 2013 and June 2013. We
253 searched the online database, *Web of Science*, using the terms 'immunocompetence
254 handicap hypothesis', 'testosterone AND immun*', 'testosterone AND parasit*',
255 'estrogen AND immun*', and 'estrogen AND parasit*'. We also searched the Internet

256 *via Google Scholar* using similar terms. Since it was impossible to use truncations
257 and wildcards in *Google Scholar*, we tried to use as many variants of a word as
258 possible to maximise our search potential. For example, we used the terms 'parasite'
259 and 'parasitic' when 'parasit*' was not possible. We included studies reported in
260 Roberts *et al*. (2004). We also included relevant studies citing Roberts *et al*. (2004).
261 Fig. 1 summarises the process and outcome of the literature search.
262
263 **(2) Inclusion/exclusion criteria**
264 Studies were included if they fulfilled the following inclusion/exclusion criteria: (1)
265 Studies must have been *in vivo*; *in vitro* studies and studies with simulated data were
266 not included. (2) Experimental studies must have manipulated hormone levels and
267 measured immune function post-manipulation. (3) Correlational studies must have
268 measured both circulating hormone levels and immune function. (4) The immune
269 function measures used must have been physiological measures of immune
270 parameters or measures of parasite loads. (5) The individuals tested in the studies
271 must have been adults. This criterion was used to prevent any confounds due to age.
272 (6) A paper must have contained extractable data (i.e. effect size and sample size
273 values or statistics that allowed us to infer these values). For relevant papers that did
274 not contain extractable data, we contacted the authors for their original data sets.
275 These papers were excluded if we could not contact the authors (see Fig. 1 for further
276 information). (7) Laboratory animals that had anomalous immune function due to
277 artificial selection (e.g. certain strains such as MRL+/+ mice) were excluded from the
278 meta-analyses. References for the included and excluded studies can be found in the
279 main reference list. Reasons for exclusion of studies are given as online supporting

information in Table S1. The final data set, including citations to the included studies, is uploaded as part of the electronic supplementary material.

(3) Effect size extraction/calculation

We chose Pearson's *r* as our measure of effect size. However, *r* is not suitable for parametric analyses because it is bounded at –1 and 1, not conforming to a normal distribution (Hedges & Olkin, 1985). Therefore, for the purpose of the statistical analyses, all extracted statistics were converted to Fisher's *Z* (hereafter termed *Zr*), which is normally distributed. All results were back-transformed to *r* to facilitate interpretations.

For experimental studies, most statistics extracted were means and variance (or uncertainty) estimates (i.e. S.D. and S.E.). For studies that reported the means and variance estimates in the form of graphs, we extracted values using the software Graphclick (Arizona-Software 2008). For studies that took multiple measures of immune function across time, we took the means and variance estimates at the time point where both conditions showed peak immune function. If the peak immune function could not be determined or if the treatment and control groups peaked at different time points, we took the means and variance estimates at the time point where the difference in immune function between the treatment and control groups was the largest. We reasoned that such differences are most likely to be biologically significant. For multifactorial studies that contained a sex-hormone-only group and a control group, we focused on the difference between the two groups. For multifactorial studies that did not have a sex-hormone-only group and a control group, we collapsed the other factors and took the results from the main effect of sex

hormone. For studies that did not report the means and variance estimates, we extracted statistics such as proportion infected, *F* values, *t* values, and *P* values.

For correlational studies, most statistics extracted were correlation coefficients (i.e. Pearson's *r*). For studies that did not report correlation coefficients, we extracted statistics such as *F* values, *P* values, Spearman's *rho*, Kendall's *tau*, β values, R^2 values, and χ^2.

All extracted statistics were converted to *Zr* based on the equations in Lipsey & Wilson (2001) using the online calculator on the Campbell Collaboration website. For experimental studies that had more than one dosage group, we combined the treatment groups into one effect size using the function to combine subgroups on the Campbell Collaboration website. We expect the effect estimates for parasite loads to be in the opposite direction to those for measures of immunity. Therefore, for the purpose of the meta-analysis, we reversed the sign of parasite load effect sizes to standardize the direction of all effect sizes. A positive effect size indicates one of the following: a stronger immune response, an increase in the baseline immune level, or a decrease in parasite load.

(4) Coding of papers

For each effect size, we recorded the species, sample size, and study identity. We also recorded the following moderator variables.

(1) Mating system: the species were classified into 'monogamous' *versus* 'polygamous' species based on published information (see Table S2). Monogamous and socially monogamous species, where individuals maintain a long-term pair bond while engaging in extra-pair copulations occasionally, were classified as 'monogamous'. Polygynous, polyandrous, polygynandrous, or lekking species were

329 classified as ‘polygamous’. We could not find information about the mating systems
330 of four species. For these species, we consulted the authors of the studies and sought
331 their expert opinions. One species was classified this way. The mating systems of the
332 other three species were treated as missing values because we were unable to contact
333 the authors. Socially monogamous species such as *Passer domesticus, Sturnus*
334 *vulgaris, and Homo sapiens* are most likely intermediate in terms of the level of
335 sexual selection they experience. Therefore, to check the robustness of our results, we
336 reclassified these three species as polygamous and re-ran those analyses that
337 contained these three species, which include the experimental and correlational
338 studies of testosterone. Our conclusions remained the same after this reclassification.
339 (2) Natural *versus* laboratory populations: species sampled from the wild were
340 classified as ‘natural’ while laboratory strains of rats, mice, and guinea pigs were
341 classified as ‘laboratory’.
342 (3) Immune measure: the immune measures were classified into ‘cell-mediated’,
343 ‘cytokines’, ‘humoral-mediated’, ‘white blood cells’, and ‘parasite load’ measures.
344 ‘Parasite load’ contained both ectoparasites and endoparasites. Our initial analyses
345 showed that the results for both types of parasite loads were very similar in all four
346 meta-analyses. The two types of parasites were therefore combined into one category.
347 (4) Immune challenge: the immune measures were classified into ‘baseline’ and
348 ‘immune reactivity’ measures.
349 (5) Gonadectomy, i.e. castration for males and ovariectomy for females
350 (experimental studies only): experimental studies were classified based on whether or
351 not gonadectomy was performed.
352 (6) Dosage (experimental studies only): hormone dosages were classified into
353 ‘physiological’ and ‘supraphysiological’ levels based on the interpretations of the

authors. Five testosterone and five oestrogen studies were treated as missing values because we could not find any information on the dosages.

(5) Building phylogenies

Using the statistical program R 3.0.3 (R Core Team, 2014), we created the phylogeny for each meta-analysis following Lim, Senior & Nakagawa (2014). We first created one main tree containing the species from all four meta-analyses using the Interactive Tree of Life online tree generator (iTOL)(http://itol.embl.de/), which generates trees based on data from the National Center for Biotechnology Information taxonomy database. Polytomies on the main tree were resolved using published phylogenies (Fabre *et al*., 2012; Leache, 2009; Mayer & Pavlicev, 2007; Pyron, Burbrink & Wiens, 2013). We then created the sub-trees for each meta-analysis by trimming the main tree, leaving only the species from that particular meta-analysis (see Figs S1–S4). Given the wide variety of species in our data sets, it was difficult to estimate the branch lengths accurately. Therefore, we only used the topology of the sub-trees (i.e. the evolutionary relationship among the species without branch-length estimates) for our meta-analyses. Using the *compute.brlen* command with the default setting of *rho* = 1 from the R package *ape*, we converted the sub-trees to an ultrametric form so that the phylogenetic correlation could be incorporated into our meta-analyses.

(6) Meta-analyses

All analyses were conducted using the statistical program R 3.0.3 (R Core Team, 2014). Using the *metafor* package (Viechtbauer, 2010), we ran multilevel meta-analyses using linear mixed models (Nakagawa & Santos, 2012; Viechtbauer,

379 2010). Linear mixed models allow us to control for non-independence in the data
380 arising due to multiple effect sizes originating from the same study, multiple effect
381 sizes originating from the same species, and shared ancestry among species (i.e.
382 phylogenetic relationship – species that are more closely related may show more
383 similar effects compared to species that are less closely related, thus resulting in non-
384 independence in the data structure), by including study identity, species identity, and
385 phylogeny into our models as random factors.

386 For each meta-analysis, we first checked the statistical significance of the
387 random variables study identity and species identity using likelihood ratio tests. Each
388 random variable was first entered into an intercept-only model (i.e. meta-analysis) and
389 tested for statistical significance. Both variables were then entered simultaneously
390 into the intercept-only model to check whether each variable had a significant effect
391 after accounting for the other. A random variable was included in subsequent models
392 if it had a significant effect and had a significant effect over and above that of the
393 other random variable. We then tested the overall effect size by running an intercept-
394 only model using restricted maximum likelihood (REML) estimation with the
395 selected random variable/s.

396 We also tested whether it was necessary to control for similarity between
397 species due to common phylogenetic descent by including phylogeny into the
398 intercept model as a random effect. If controlling for phylogeny influenced the
399 magnitude and/or significance of our overall effect size, phylogeny was included as a
400 random effect for all subsequent analyses. If not, subsequent analyses were run
401 without phylogeny.

402 We computed the heterogeneity statistic I^2 by running an intercept-only model
403 without any random effects using the *rma* function in *metafor*. The statistic I^2 tells us

404 the percentage of variability in the effect sizes that is not due to sampling error
405 (Higgins *et al*., 2003; Higgins & Thompson, 2002). If I^2 was moderate to large (i.e. >
406 50% according to Higgins *et al*., 2003; Higgins & Thompson, 2002), we proceeded to
407 run moderator analyses.

408 Moderator analyses were conducted by running meta-regression models using
409 the *rma.mv* function in *metafor*. We first ran single-factor models without the
410 intercept using REML estimation by entering each moderator as a fixed factor
411 together with the random factors to obtain the parameter estimates of each level in
412 each factor after controlling for the random factors. We then ran an automated model
413 selection (Burnham & Anderson, 2002; Grueber *et al*., 2011) using the package
414 *MuMIn* (Barton, 2014 to identify the moderators that remained in the final model. The
415 model selection was based on the Akaike Information Criterion with sample size
416 correction (AICc; Anderson, 2008; Burnham & Anderson, 2002) obtained from
417 maximum likelihood (ML) estimation. We ran the model selection using only the
418 effect sizes that had no missing data to ensure that the AICc values of the different
419 models were comparable (Nakagawa & Freckleton, 2011). We first generated all the
420 possible models from the moderator variables in the data set. We then averaged the
421 model coefficients (without shrinkage) of all models within two AICc units from the
422 best model, indicated by having the lowest AICc unit. We tested the significance of
423 the moderators that were retained in the final averaged models using the Q test. The Q
424 test provides an omnibus test for each moderator. It is also more conservative
425 compared to testing the Z values derived from the model estimates. We interpreted the
426 variables in the final averaged model using the parameter estimates obtained from the
427 single-factor models.
428

(7) Publication bias

We analysed the relationship between year of publication and effect size for potential time-lag bias. Time-lag bias is the tendency for some studies to be published faster than others depending on the direction and magnitude of their results, usually with studies having large effects being published first (Jennions & Møller, 2002). We first ran a single-factor meta-regression model with year of publication entered as a moderator together with the random factors using the *rma.mv* function in *metafor*. We then entered year of publication together with the other moderators into the AICc model selection to see whether it was retained in the final averaged model and, if it was, whether it significantly predicted effect size after controlling for the rest of the fixed and random factors.

We looked for potential missing effect sizes by running two funnel plot asymmetry analyses using the meta-analytic residuals (*sensu* Nakagawa & Santos, 2012) extracted from the final averaged model using the *MCMCglmm* function in the *MCMCglmm* package (Hadfield, 2010); note that the residuals incorporating random effects could only be extracted from the models using *MCMCglmm* but not *metafor*. We used 130,000 iterations, 100 thinning, 30,000 burn-in, and inverse gamma prior for all four residual extractions. We ran Egger's regression test (Egger *et al*., 1997) on the residuals using the *regtest* function in *metafor*. Egger's test regresses the standardised residuals on precision. Publication bias is indicated by an intercept that is significantly different from zero.

We ran a trim-and-fill analysis (Duval & Tweedie, 2000) on the residuals using the *trimfill* function in *metafor* to test for funnel plot asymmetry and identify missing studies. The analysis assumes that the funnel plot is symmetric and attempts to remove (trimming) the smaller studies that are causing asymmetry while filling the

distribution with missing studies to symmetrize the distribution. We also used the I^2 statistic reported in the *trimfill* function to look at the amount of heterogeneity left after accounting for the random and fixed factors that were retained in the final averaged model.

III. RESULTS

(1) Testosterone

The results (parameter estimates) of the meta-analytic and meta-regression models for both experimental and correlational studies are presented in Fig. 2 and Table 1.

(*a*) *Experimental studies*

Both study identity (likelihood ratio test: $\chi^2_1 = 486.17$, $P < 0.0001$) and species identity (likelihood ratio test: $\chi^2_1 = 110.83$, $P < 0.0001$) significantly improved the model when entered individually into the model, but species identity did not have a significant effect over and above the effect of study identity (likelihood ratio test: $\chi^2_1 = 0$) when both random variables were entered into the model simultaneously. Therefore, we only included study identity as the random variable in subsequent analyses. Overall, there was a medium significant immunosuppressive effect of testosterone after controlling for study identity ($r_{overall} = –0.28$, 95% CI [–0.39, –0.17], $P < 0.0001$; Fig. 2A). Controlling for similarity due to common phylogenetic descent did not have a significant effect on the effect size (likelihood ratio test: $\chi^2_1 = 0$). Therefore we ran all subsequent analyses without controlling for phylogeny.

There was large heterogeneity in this data set (I^2 = 89.16%). Therefore we conducted moderator analyses. The final averaged model from the AICc model selection retained the factors castration and immune measure (see Table S3 for model results). There was a significant effect of castration (Q_1 = 5.55, P = 0.02). The immunosuppressive effect was stronger in castrated animals, which showed a significant medium-to-large negative effect ($r_{castrated}$ = –0.41, 95% CI [–0.55, –0.24], P < 0.0001), compared to non-castrated animals, which showed a small-to-medium negative effect, ($r_{non\text{-}castrated}$ = –0.22, 95% CI [–0.35, –0.07], P = 0.004) (Fig. 2A). The effect of immune measure was non-significant (Q_4 = 6.59, P = 0.16).

There was a small and significant positive relationship between year of publication and effect size in the single-factor model (slope estimate = 0.03, 95% CI [0.01, 0.04], P = 0.001) (Fig. 3). Year of publication was also retained in the final AICc model, but the relationship between year of publication and effect size became non-significant after controlling for immune measure and castration (Q_1 = 3.65, P = 0.06). Therefore, the temporal trend appears to be due to changes in moderators over time.

For the two funnel plot analyses, Egger's regression test did not indicate significant asymmetry in the funnel plot of the residuals (t_{154} = –0.83, P = 0.41). The trim-and-fill analysis estimated a total of 34 effect sizes missing from the right side of the distribution and that the effect-size estimate should be adjusted to r = –0.15 (95% CI [–0.26, –0.04]) (Fig. 4A), indicating that the actual effect of testosterone might be smaller than our initial estimate of r = –0.28. The I^2 statistic indicated that considerable heterogeneity still remained in the residuals (81.11%), suggesting that the effect of testosterone might be moderated by other variables.

(*b*) *Correlational studies*

Both study identity (likelihood ratio test: $\chi^2_1 = 117.27$, $P < 0.0001$) and species identity (likelihood ratio test: $\chi^2_1 = 42.84$, $P < 0.0001$) significantly improved the model when entered individually into the model, but species identity did not have a significant effect over and above the effect of study identity (likelihood ratio test: $\chi^2_1 = 0$) when both random variables were entered into the model simultaneously. Therefore, we only included study identity as the random variable in subsequent analyses. Overall, the correlation between circulating testosterone level and immune function after controlling for study identity was small and non-significant (Fig. 2B; Table 1). Controlling for similarity due to common phylogenetic descent did not have a significant effect on the effect size (likelihood ratio test: $\chi^2_1 = 0$). Therefore we ran all subsequent analyses without controlling for phylogeny.

There was large heterogeneity in this data set ($I^2 = 95.41\%$). Therefore we conducted moderator analyses. The final averaged AICc model retained the moderators immune measure, immune challenge, and mating system (see Table S4 for model results). There was a significant effect of immune measure ($Q_3 = 8.56$, $P = 0.04$). There was a medium positive relationship between cell-mediated immune function and testosterone that was significant ($r_{\text{cell-mediated}} = 0.26$, 95% CI [0.007, 0.48], $P = 0.04$) (Fig. 2B). The relationship between immune function and testosterone was non-significant for the other immune measures ($r_{\text{humoral-mediated}} = 0.06$, 95% CI [–0.16, 0.27], $P = 0.59$; $r_{\text{parasite load}} = –0.17$, 95% CI [–0.50, 0.21], $P = 0.38$; $r_{\text{white blood cells}} = 0.08$, 95% CI [–0.14, 0.29], $P = 0.47$). The effect of immune challenge ($Q_1 = 2.46$, $P = 0.12$) and mating system ($Q_1 = 0.40$, $P = 0.53$) were non-significant.

No indirect evidence of publication bias was detected. The relationship between year of publication and effect size was non-significant in the single factor model

527 (slope estimate = –0.02, 95% CI [–0.05, 0.01], $P = 0.17$). Year of publication was
528 retained in the final averaged AICc model, but the relationship between year and
529 effect size remained non-significant after controlling for immune measure, immune
530 challenge, and mating system ($Q_1 = 2.62$, $P = 0.11$). Egger's regression test indicated
531 no significant asymmetry in the funnel plot of the residuals ($t_{107} = 0.18$, $P = 0.85$).
532 Trim and fill analysis estimated no missing effect sizes (Fig. 4B). The I^2 statistic
533 indicated that only a small amount of heterogeneity remained in the residuals
534 (20.84%).
535
536 **(2) Oestrogen**
537 The results (parameter estimates) of the meta-analytic and meta-regression
538 models for both experimental and correlational studies are shown in Fig. 5 and Table
539 2.
540
541 (*a*) *Experimental studies*
542 When entered individually into the model, both study identity (likelihood ratio
543 test: $\chi^2_1 = 911.63$, $P < 0.0001$) and species identity (likelihood ratio test: $\chi^2_1 = 11.11$, P
544 $= 0.0009$) significantly improved the model, but species identity did not have a
545 significant effect over and above the effect of study identity (likelihood ratio test: χ^2_1
546 $= 0$) when both random variables were entered into the model simultaneously.
547 Therefore we only included study identity as the random variable in subsequent
548 analyses. Overall, the effect of oestrogen on immune function after controlling for
549 study identity was small and non-significant (Fig. 5A; Table 2). Controlling for
550 similarity due to common phylogenetic descent did not have a significant effect on

551 effect size (likelihood ratio test: $\chi^2_1 = 0$). Therefore we ran all subsequent analyses
552 without controlling for phylogeny.
553 There was large heterogeneity in this data set ($I^2 = 94.60\%$). Therefore, we
554 conducted moderator analyses. The final averaged AICc model retained the following
555 moderators: immune measure, immune challenge, ovariectomy, and dosage (see
556 Table S5 for model results). There was a significant effect of immune measure ($Q_4 =$
557 41.28, $P < 0.001$). Oestrogen had a medium-to-large immunosuppressive effect on
558 cell-mediated immune function that was significant ($r_{\text{cell-mediated}} = –0.41$, 95% CI [–
559 0.65, –0.09], $P = 0.01$). The effects of oestrogen on parasite load, humoral-mediated
560 immune function, and cytokine levels were in the opposite direction. Oestrogen had a
561 medium-to-large immunoenhancing effect on parasite load (i.e. reducing parasite
562 load) that was significant ($r_{\text{parasite load}} = 0.46$, 95% CI [0.09, 0.72], $P = 0.02$).
563 Oestrogen also had a medium but non-significant immunoenhancing effect on
564 humoral-mediated immune function and cytokine level ($r_{\text{humoral-mediated}} = 0.30$, 95% CI
565 [–0.02, 0.56], $P = 0.07$; $r_{\text{cytokine}} = 0.29$, 95% CI [–0.04, 0.56], $P = 0.08$) (Fig. 5A;
566 Table 2). There was a significant effect of immune challenge ($Q_1 = 40.61$, $P < 0.001$).
567 Studies using measures of baseline immunity showed a large positive effect that was
568 significant ($r_{\text{baseline immunity}} = 0.60$, 95% CI [0.11, 0.85], $P = 0.02$) while studies using
569 measures of immune reactivity showed a small and non-significant effect (r_{immune}
570 $_{\text{reactivity}} = 0.10$, 95% CI [–0.22, 0.40], $P = 0.54$) (Fig. 5A; Table 2). There was a
571 significant effect of dosage ($Q_1 = 10.36$, $P = 0.001$). Studies using supraphysiological
572 oestrogen dosages showed a medium-to-large positive effect that was significant
573 ($r_{\text{supraphysiological}} = 0.48$, 95% CI [0.14, 0.72], $P = 0.008$) while studies using
574 physiological dosages showed a small and non-significant effect ($r_{\text{physiological}} = –0.05$,

95% CI [–0.40, 0.31], $P = 0.78$) (Fig. 5A; Table 2). The effect of ovariectomy ($Q_1 = 1.82$, $P = 0.18$) was non-significant

There was a small but significant positive relationship between year of publication and effect size in the single-factor model (estimate = 0.04, 95% CI [0.004, 0.08], $P = 0.03$), but the relationship was highly influenced by one large negative effect size in 1974 (Fig. 6). We re-ran the time-lag bias analysis a second time excluding that particular effect size. The relationship became non-significant (estimate = 0.02, 95% CI [–0.02, 0.06], $P = 0.30$). Year of publication was retained in the final averaged AICc model, but the relationship between year and effect size remained non-significant after controlling for immune measure, immune challenge, ovariectomy, and dosage ($Q_1 = 3.70$, $P = 0.054$). Therefore, there was no evidence of a significant temporal trend.

Egger's regression test indicated no significant asymmetry in the funnel plot ($t_{126} = 0.51$, $P = 0.61$). The trim-and-fill analysis estimated a total of 31 effect sizes missing from the left side of the distribution. However, the missing effect sizes did not qualitatively influence the results. The overall effect size estimate remained small and non-significant after adjusting for the missing effect sizes (–0.10, 95% CI [–0.41, 0.18]) (Fig. 7A). I^2 indicated considerable heterogeneity in the residuals (89.23%).

(*b*) *Correlational studies*

Out of the 64 effect sizes in this data set, 60 effect sizes came from the same species and a single study while the remaining four effect sizes came from two species and three other studies. It was therefore impossible to distinguish between the variances of study identity and species identity. Therefore we only tested study identity as a random factor. Study identity significantly improved the model

600 (likelihood ratio test: $\chi^2_1 = 7.99$, $P < 0.0001$) and was included as a random variable
601 for subsequent analyses. Overall, there was a significant medium-to-large positive
602 relationship between circulating oestrogen level and immune function after
603 controlling for study identity ($r_{overall} = 0.43$, 95% CI [0.11, 0.66], $P = 0.01$) (Fig. 5B;
604 Table 2). Controlling for similarity due to common phylogenetic descent did not have
605 a significant effect on the effect size (likelihood ratio test: $\chi^2_1 = 0.57$, $P = 0.45$).
606 However, the overall effect size became non-significant after controlling for
607 phylogeny ($r_{phylogenetic} = 0.42$, 95% CI [–0.17, 0.79], $P = 0.16$) (Fig. 5B; Table 2). We
608 therefore ran all subsequent analyses with phylogeny included as a random variable.
609 There was moderate heterogeneity in this data set ($I^2 = 60.52\%$). Therefore, we
610 conducted moderator analyses. We could not perform an AICc model selection
611 because immune measure was confounded with immune challenge: all of the cell-
612 mediated and cytokine measures were reactivity measures and all of the humoral-
613 mediated and white blood cell measures were baseline measures. Therefore, we ran
614 single-factor moderator analyses for the two factors separately. Neither the effects of
615 immune measure ($Q_3 = 1.16$, $P = 0.76$) nor immune challenge ($Q_1 = 0.06$, $P = 0.80$)
616 were significant (Fig. 5B; Table 2).
617 Year of publication was not significantly related to effect size in the single-
618 factor model (estimate = –0.05, 95% CI [–0.13, 0.04], $P = 0.27$). Since none of the
619 moderator effects were significant, we extracted the residuals from the intercept-only
620 model with study identity and phylogeny entered as random factors. Egger's
621 regression test indicated no significant asymmetry in the residual funnel plot ($t_{62} = –$
622 0.19, $P = 0.85$). Trim-and-fill analysis estimated no missing effect sizes (Fig. 7B). I^2
623 indicated moderate heterogeneity in the residuals (56.22%).
624

IV. DISCUSSION

(1) The relationship between testosterone and immune function in males

The results from the experimental studies support the hypothesis that testosterone suppresses immune function (Ansar Ahmed *et al*., 1985; Bouman *et al*., 2005; Grossman, 1985; Klein, 2004; Schuurs & Verheul, 1990). Overall, testosterone manipulation had a medium immunosuppressive effect ($r = –0.28$) that was significant. Controlling for similarity due to common phylogenetic descent did not influence our results, which suggests that our results may be generally applicable across the species studied.

Castrated animals showed a stronger immunosuppressive effect than non-castrated animals. One possible explanation is that in the non-castrated animals, testosterone manipulation in the treatment group triggered a compensatory reduction in endogenous testosterone *via* a feedback loop, thus reducing the difference in testosterone levels between the treatment and control groups.

Our results provide important support for a critical assumption of the ICHH (Folstad & Karter, 1992), namely that testosterone suppresses immune function. According to the ICHH, testosterone-based male ornaments are honest signals of immune function because the immunosuppressive effect of testosterone makes it impossible for males with poor immune function to sustain high levels of testosterone for ornamentation. In their meta-analysis, Roberts *et al*. (2004) found that the overall effect of testosterone on immune function in males was non-significant and that a significant immunosuppressive effect was found only in reptiles and not in mammals or birds. They concluded that there was little support for the ICHH. Thus, our results differ from those of Roberts *et al*. (2004). They found an overall effect of $d = –0.32$, which transforms to $r = –0.16$. Our overall effect size ($r = –0.28$) was almost twice

650 that. The difference in findings is probably due to the accumulation of studies since
651 2004 and the inclusion of studies from fields other than evolutionary biology, such as
652 biomedical sciences, in our meta-analysis. Based on our results, there is good
653 evidence that testosterone suppresses immune function.

654 We assumed that the effect of testosterone would be stronger in polygamous
655 species compared to monogamous species. Polygamous species face more intense
656 sexual selection and are thus expected to evolve stronger condition dependence of
657 ornamentation (Andersson, 1994). However, mating system did not influence the size
658 of the effect of testosterone on immune suppression. One possible reason for a lack of
659 a mating-system effect might be that, while in polygamous systems a trade-off
660 between testosterone and immune function is driven by male expenditure on
661 ornaments, in monogamous systems the same trade-off is driven by male expenditure
662 on parental care. Indeed, there is evidence that male parental care is associated with a
663 reduction in circulating testosterone level (Gray, Yang & Pope, 2006; Wingfield *et*
664 *al*., 1990). It is possible then that we did not observe a significant effect of mating
665 system because trade-offs between mating and parental care may balance the overall
666 reproductive effort among polygamous and monogamous species.

667 In contrast to the experimental studies, we did not find a significant overall
668 correlation between testosterone and immune function. Our results are consistent with
669 arguments that correlational studies are not ideal for testing the effect of testosterone
670 on immune function (Getty, 2006).

671

672 **(2) The relationship between oestrogen and immune function in females**

673 The effect of oestrogen manipulation on immune function depended on the
674 immune measure used. Oestrogen had a significant medium-to-large

675 immunosuppressive effect on cell-mediated immune function ($r_{\text{cell-mediated}} = -0.41$) but
676 had a significant medium-to-large immunoenhancing effect on parasite loads (r_{parasite}
677 $_{\text{load}} = 0.46$). Oestrogen also had a medium but non-significant immunoenhancing
678 effect on humoral-mediated immune function and cytokine level. Although these
679 effects on humoral-mediated immune function and cytokine level were non-
680 significant, the effect sizes ($r_{\text{humoral-mediated}} = 0.30$ and $r_{\text{cytokine}} = 0.29$) were medium in
681 magnitude (Cohen, 1988). Moreover, the effect sizes were slightly larger than those
682 typically found in biological studies, which range from $r = 0.16$ to 0.25 (Møller &
683 Jennions, 2002). Therefore, the immunoenhancing effect of oestrogen on humoral-
684 mediated immune function and cytokine level may prove to be biologically important
685 despite the lack of statistical significance.

686 Our results are consistent with observations by researchers that oestrogen
687 suppresses cell-mediated immune function and enhances humoral-mediated immune
688 function (Cutolo *et al*., 1996; Klein, 2004). However, little is known about why
689 oestrogen would have different effects on different immune components. This
690 diversity in effects across immune components may reflect life-history trade-offs in
691 females based on the costs and benefits of different immune components (Lee, 2006).
692 Cell-mediated responses are energetically and nutritionally costly because they are
693 associated with the activation of the systemic inflammatory response (Halloran *et al*.,
694 1992; Janeway *et al*., 1999). In comparison, humoral-mediated responses are less
695 costly because they are associated with the activation of the anti-inflammatory system
696 (Janeway *et al*., 1999). Lee (2006) argued that because females invest more energy
697 and resources in their offspring compared to males (Trivers, 1972), females are
698 expected to adopt an immune profile that is less cell-mediated and more humoral-
699 mediated. Doing so allows females to reduce the cost of maintaining a healthy

immune system while diverting energetic resources towards reproduction and parenting. Our results suggest that oestrogen, the major female sex hormone, influences the immune profile females adopt by suppressing cell-mediated immune function and enhancing humoral-mediated immune function.

We also found that the effect of oestrogen depended on whether measures of baseline immunity or immune reactivity were used. Given that measures of immune reactivity are considered more rigorous measures of immune function compared to baseline immunity (Demas *et al*., 2011; Norris & Evans, 2000), it is surprising to find that immune reactivity showed a small and non-significant effect ($r_{\text{immune reactivity}} = 0.10$) while baseline immunity showed a large positive and significant effect ($r_{\text{baseline immunity}} = 0.60$). However, it should be noted that the different measures of immune function were not equally distributed between immune reactivity and baseline immunity. Baseline immunity consisted of only white blood cell and humoral-mediated measures (19 effect sizes in total), while immune reactivity consisted of all five immune measures (127 effect sizes in total). As discussed above, the effect of oestrogen on immune function depends on the immune measure. Therefore, the non-significant results for immune reactivity may be due to the effects of the different immune measures cancelling each other out. On the other hand, the large effect size for baseline immunity may be due to a relatively small sample of effect sizes that consists of immune measures on which oestrogen had a positive effect. Nonetheless, it remains possible that the effect of oestrogen on immune function could differ for baseline and reactivity measures of immune function.

Besides moderators associated with the measurement of immune function, there was also an effect of dosage. Larger dosages showed larger effects. Specifically, supraphysiological dosages led to a significant medium-to-large immunoenhancing

725 effect ($r_{supraphysiological}$ = 0.48) while physiological dosages showed a non-significant
726 effect that was close to zero ($r_{physiological}$ = –0.05).
727 Overall, there was a medium-to-large positive relationship in correlational
728 studies between circulating oestrogen level and immune function (r = 0.43), but this
729 relationship became non-significant after we controlled for the similarity due to
730 common phylogenetic descent between species. This change was due largely to the
731 widening of the confidence intervals after accounting for phylogeny. This data set
732 consists of 64 effect sizes, 60 of which belonged to one single study of one species.
733 Therefore, the widening of the confidence interval probably reflects the over-
734 representation of a single species in the data set and the meta-analytic mean may not
735 be general. Furthermore, unlike the results from the experimental studies, the
736 correlation between oestrogen and immune function did not depend on immune
737 measure or immune challenge. Like the testosterone results, the oestrogen results
738 suggest that correlational designs are unsuitable for testing the effects of sex
739 hormones on immune function.
740
741 **(3) Publication bias**
742 We found a significant positive relationship between year of publication and
743 effect size for experimental studies of testosterone. Decreases in the magnitude of
744 effect sizes over time have been reported in numerous meta-analyses in evolutionary
745 biology (Jennions & Møller, 2002). It should, however, be noted that the analyses
746 used in this study are indirect tests of publication bias. A direct test of publication bias
747 requires a comparison of the effect sizes between published and unpublished studies
748 (Song *et al*., 2000; Møller & Jennions, 2001). A significant result from indirect tests
749 may not always indicate publication bias (Jennions & Møller, 2002; Jennions *et al*.,

2013; Koricheva, Jennions & Lau, 2013). For example, the temporal trend we found for experimental studies of testosterone appears to be due to changes in moderators across time. We found that the significant trend disappeared after we controlled for castration and immune measure. We graphically explored the moderators that changed across time by plotting the relationship between year of publication and effect size using different colours for each moderator level (Figs S5 and S6). We found that in the later years, more studies were conducted using cell-mediated and cytokine measures. The effect of testosterone on cytokine levels is smaller than the overall effect size (Fig. 2A; Table 1). The effect size for cell-mediated measures seems to be comparable to the overall effect size (Fig. 2A; Table 1). Therefore, the significant temporal trend seems likely to have been due to the increase in number of effect sizes assessing cytokines in recent years. Castration was fairly equally distributed across year of publication. Our finding suggests that the significant temporal trend was not due to a publication bias.

The trim-and-fill analysis also detected a substantial number of missing effect sizes in the same data set. Although the missing effect sizes did not change the results qualitatively, they did reduce the overall effect size by almost half from a medium effect size ($r = –0.28$) to a small effect size ($r = –0.15$). Even though the immunosuppressive effect remained significant, our result suggests that the effect of testosterone on immune function might not be as strong as initially indicated. However, like the temporal trend findings, caution must be exercised when interpreting the results from the trim-and-fill analysis because the findings might reflect causes other than publication bias (Thornhill, Møller & Gangestad, 1999; Jennions *et al*., 2013). Heterogeneity in effect sizes can also lead to funnel plot asymmetry. We tried to control for the effects of heterogeneity by running the trim-

775 and-fill analysis on the residuals extracted from the final AICc model. However, we
776 found that the I^2 value for experimental studies remained high even after we
777 controlled for the random and moderator variables. Therefore, the funnel plot
778 asymmetry we observed in the experimental studies might have been caused by
779 unidentified moderators and not by publication bias. We therefore believe that the
780 initial estimate of $r = –0.28$ is more reflective of the actual effect size.
781 We detected little indirect evidence of publication bias for the other three data
782 sets. The trim-and-fill analysis estimated 32 missing effect sizes from the oestrogen
783 experimental studies, but the missing effects did not influence the results. Overall, our
784 results seem fairly robust to publication bias.
785
786 **(4) Sex differences in immune function**
787 Females tend to have better immune function compared to males (i.e. lower and
788 less-intense parasitism and stronger immune responses) (Klein, 2004; Schuurs &
789 Verheul, 1990; Zuk & McKean, 1996). Our results suggest that these sex differences
790 might be due to the combined effects of testosterone in males and oestrogen in
791 females. Our results also showed that the effect for oestrogen depends on the immune
792 measure. Therefore, it would be interesting to examine studies looking at sex
793 differences in immune function and test whether the effect sizes differ depending on
794 the immune measure.
795
796 **(5) Heterogeneity in the effects of sex hormones on immune function**
797 In meta-analysis, it is important to examine both the mean effect size and the
798 variance of the effect sizes (i.e. heterogeneity). The main tenet of life-history theory is
799 that trade-offs between fitness components occur due to limited resource availability.

One implication of this theory is that trade-offs between fitness components could vary across individuals. Therefore, one would predict that the effect of sex hormones, which mediate trade-offs between immune function and reproductive functions, would show significant heterogeneity. Indeed, we found large heterogeneity in the effect sizes across all four data sets. We ran moderator analyses to examine the factors that account for the variation in effect sizes. However, the heterogeneity remained moderate to large for three of the analyses, apart from correlational studies of testosterone, even after we accounted for the random and moderator effects from the final averaged AICc models.

Increasing evidence suggests that the effects of sex hormones on immune function can be dependent on individual condition. The amount of resources available to individuals varies substantially. It has been predicted that trade-offs between fitness components occur only when resources are limiting (van Noordwijk & de Jong, 1986; McDade, 2003). For example, the effect of testosterone on immune function in *Sceloporus graciosus* lizards depends on the quality of food available to the lizards (Ruiz *et al*., 2010). Testosterone enhanced immune function in lizards that were given extra vitamins on top of their usual diet, but decreased immune function in lizards that did not receive extra vitamins. The effect of testosterone might also depend on the effect of leptin, a hormone that functions as a signal of energetic resource level. In a study on zebra finches (*Taeniopygia guttata*), leptin increased immune function and prevented the immunosuppressive effect of testosterone (Alonso-Alvarez *et al*., 2007*b*). The effect of testosterone on immune function may also depend on stress levels [Rantala *et al*., 2012, but see Roberts *et al*. (2009) and Roberts *et al*. (2007*a*) for contradictory findings].

In relation to the issue of looking at variance, recent advances have applied meta-analytic techniques to analysing the variance instead of the mean data of experimental studies involving two groups (Nakagawa *et al*., 2015). For example, instead of asking whether testosterone suppresses immune function, we could ask whether testosterone increases or decreases the variance in immune function across individuals relative to controls. We did not run such analyses because the theoretical predictions were focused on the mean effect and not the variance.

(6) Limitations and future directions

Sex hormone levels change across time in response to life-history changes. For example, testosterone in males peaks during the breeding season and drops when the breeding season ends (Wingfield *et al.*, 1990; Nelson, 2005). Similarly, oestrogen in females varies across the fertility cycle (Abraham *et al*., 1972). One might wonder how relevant the results in this meta-analysis are for understanding the effects of sex hormones during different life-history stages. In this review, for the testosterone studies, all studies except five that were unclassified reported using physiological dosages. For the oestrogen studies, we found a significant effect of immune measure even after controlling for the effect of dosage. Therefore, we were able to conclude that our results were not just an artefact of using dosages that were in excess of what is normally found in the body. However, we were unable to look at the seasonal or life-history relevance of the dosage levels because most studies did not provide such information. Future studies examining the effects of sex hormones in relation to different life-history stages will provide us with a better understanding of the effects of sex hormones on the immune system.

848 The studies reviewed herein have focused on the strength of the immune
849 response. Navarro *et al*. (2003) found a positive correlation between immune-
850 response strength and latency to maximum immune response in *Passer domesticus*.
851 Their results suggest a trade-off between the strength and rapidity of the immune
852 response. Therefore, future studies should measure both the strength and time course
853 of the immune response to gain a better picture of the effects of sex hormones on
854 immune function.
855

856 **V. CONCLUSIONS**

857 (1) We found meta-analytic evidence that testosterone has a medium-sized
858 suppressive effect on immune function. This effect was generalizable across the
859 species studied. Castrated animals showed a greater immunosuppressive effect than
860 non-castrated animals, but the immunosuppressive effect was significant in both
861 cases. Our overall effect size for experimental studies of testosterone was almost
862 twice that of a previous meta-analysis (Roberts *et al*., 2004).
863 (2) We also found meta-analytic evidence that oestrogen has a medium-to-large
864 suppressive effect on cell-mediated immune function while having a medium-to-large
865 effect in reducing parasite loads and a medium but non-significant enhancing effect
866 on humoral-mediated immune function and cytokine level. Oestrogen also had a
867 significant immune-enhancing effect in studies using supraphysiological dosages and
868 studies using baseline measures of immune function.
869 (3) When effect sizes were derived from correlational studies, the relationships
870 between circulating sex hormone levels and immune function measurements were
871 small and non-significant for both testosterone and oestrogen, suggesting that

872 correlational studies are unsuitable for testing the effects of sex hormones on immune
873 function. Thus, an experimental approach is imperative to study the effects of sex
874 hormones on immune function.
875 (4) We found little evidence of publication bias using indirect tests. There was a small
876 and positive relationship between year of publication and effect size for experimental
877 studies of testosterone that became non-significant after we controlled for castration
878 and immune measure, suggesting that the temporal trend was due to changes in
879 moderators over time. The trim-and-fill analysis for experimental studies of
880 testosterone estimated a total of 34 missing effect sizes and that the
881 immunosuppressive effect of testosterone should be reduced from –0.28 to –0.15.
882 However, due to the substantial heterogeneity in the residuals after accounting for the
883 random and fixed factors, we cannot rule out the possibility that the asymmetry in the
884 funnel plot was due to heterogeneity. Overall, our results seem to be fairly robust to
885 publication bias.
886 (5) We found substantial heterogeneity in the effect sizes for all four meta-analyses.
887 The amount of heterogeneity in three of the meta-analyses, apart from correlational
888 studies of testosterone, remained substantial even after we accounted for the relevant
889 random and fixed factors, suggesting that there are other factors that moderate the
890 effects of sex hormones on immune function.
891

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VIII. SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Table S1. Excluded studies with reasons for this exclusion.

Table S2. References used for mating system classification.

Fig. S1. Phylogeny for experimental studies of testosterone.

Fig. S2. Phylogeny for correlational studies of testosterone.

Fig. S3. Phylogeny for experimental studies of oestrogen.

Fig. S4. Phylogeny for correlational studies of oestrogen.

Table S3. Details of the summary statistics provided by the final averaged model from the AICc model selection for experimental studies of testosterone, including the *Q* statistics for the test of main effect significance for each moderator.

Table S4. Details of the summary statistics provided by the final averaged model from the AICc model selection for correlational studies of testosterone, including the Q statistics for the test of main effect significance for each moderator.

1756 **Table S5.** Details of the summary statistics provided by the final averaged model
1757 from the AICc model selection for experimental studies of oestrogen, including
1758 the Q statistics for the test of main effect significance for each moderator.
1759 **Fig. S5.** The relationship between year of publication and effect size for experimental
1760 studies of testosterone. Effect sizes are separated by castration: black indicates
1761 no castration; blue indicates castration.
1762 **Fig. S6.** The relationship between year of publication and effect size for experimental
1763 studies of testosterone. Effect sizes are separated by immune measure: blue indicates
1764 cell-mediated measures; light blue indicates cytokine levels; black indicates humoral-
1765 mediated measures, grey indicates parasite loads; purple indicates white blood cell
1766 counts.
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1768

Table 1. Parameter estimates and *P*-values for the effect of testosterone manipulation on immune function and the correlation between circulating testosterone level and immune function in males. *M* is the mean effect size. CI.lb and CI.ub are the lower and upper bounds of the 95% confidence interval respectively. * indicates moderators that were retained in final AICc models.

	Experimental studies					Correlational studies			
	M	CI.lb	CI.ub	*P*		*M*	CI.lb	CI.ub	*P*
Meta-analytic mean	−0.28	−0.39	−0.17	< 0.0001	**Meta-analytic mean**	0.04	−0.14	0.21	0.66
Phylogenetic mean	−0.28	−0.39	−0.17	< 0.0001	**Phylogenetic mean**	0.04	−0.14	0.21	0.66
Mating system					**Mating system***				
Polygamous	−0.30	−0.43	−0.16	< 0.0001	Polygamous	−0.001	−0.24	0.24	0.99
Monogamous	−0.17	−0.33	0.006	0.06	Monogamous	0.09	−0.18	0.34	0.51
Natural *vs* Lab					**Immune measure***				
Natural	−0.25	−0.37	−0.11	0.0004	Cell-mediated	0.26	0.007	0.48	0.04
Lab	−0.41	−0.60	−0.17	0.0011	Humoral-mediated	0.06	−0.16	0.27	0.59
Immune measure*					Parasite load	−0.17	−0.50	0.21	0.38
Cell-mediated	−0.29	−0.42	−0.14	0.0002	White blood cells	0.08	−0.14	0.29	0.47
Cytokines	−0.03	−0.34	0.29	0.87	**Immune-challenged***				
Humoral-mediated	−0.32	−0.45	−0.17	< 0.0001	Yes	0.05	−0.14	0.24	0.59
Parasite load	−0.33	−0.45	−0.18	< 0.0001	No	0.03	−0.16	0.21	0.77
White blood cells	−0.24	−0.40	−0.07	0.007					
Immune-challenged									
Yes	−0.29	−0.40	−0.16	< 0.0001					
No	−0.28	−0.42	−0.12	0.0007					
Castrated*									
Yes	−0.41	−0.55	−0.24	< 0.0001					
No	−0.22	−0.35	−0.07	0.004					

Table 2. Parameter estimates for the effect of oestrogen manipulation on immune function and the correlation between circulating oestrogen level and immune function in females. *M* is the mean effect size. CI.lb and CI.ub are the lower and upper bounds of the 95% confidence interval, respectively.* indicates moderators that were retained in final AICc models.

	Experimental studies					Correlational studies			
	M	CI.lb	CI.ub	*P*		*M*	CI.lb	CI.ub	*P*
Meta-analytic mean	0.16	−0.15	0.44	0.30	**Meta-analytic mean**	0.43	0.11	0.66	0.01
Phylogenetic mean	0.16	−0.15	0.44	0.30	**Phylogenetic mean**	0.42	−0.17	0.79	0.16
Immune measure*					**Immune measure**				
Cell-mediated	−0.41	−0.65	−0.09	0.01	Cell-mediated	0.42	−0.18	0.79	0.16
Cytokines	0.29	−0.04	0.56	0.08	Cytokines	0.48	−0.45	0.91	0.31
Humoral-mediated	0.30	−0.02	0.56	0.07	Humoral-mediated	0.5	−0.08	0.83	0.08
Parasite load	0.46	0.09	0.72	0.02	White blood cells	0.42	−0.18	0.79	0.16
White blood cells	0.10	−0.27	0.44	0.61	**Immune-challenged**				
Immune-challenged*					Yes	0.42	−0.21	0.80	0.18
Yes	0.10	−0.22	0.40	0.54	No	0.43	−0.18	0.81	0.16
No	0.60	0.11	0.85	0.02					
Ovariectomized*									
Yes	0.09	−0.24	0.40	0.59					
No	0.41	−0.05	0.72	0.08					
Dosage*									
Physiological	−0.05	−0.40	0.31	0.78					
Supraphysiological	0.48	0.14	0.72	0.008					

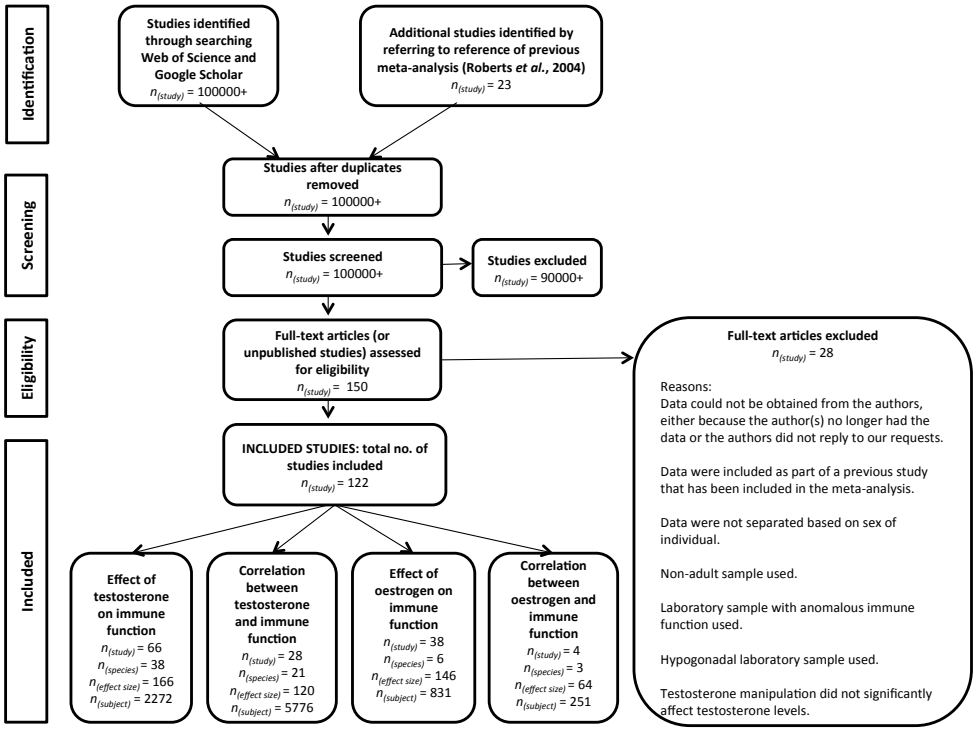

Fig. 1. Prisma flow chart (Moher *et al*., 2009) depicting the process and outcome of the literature search.

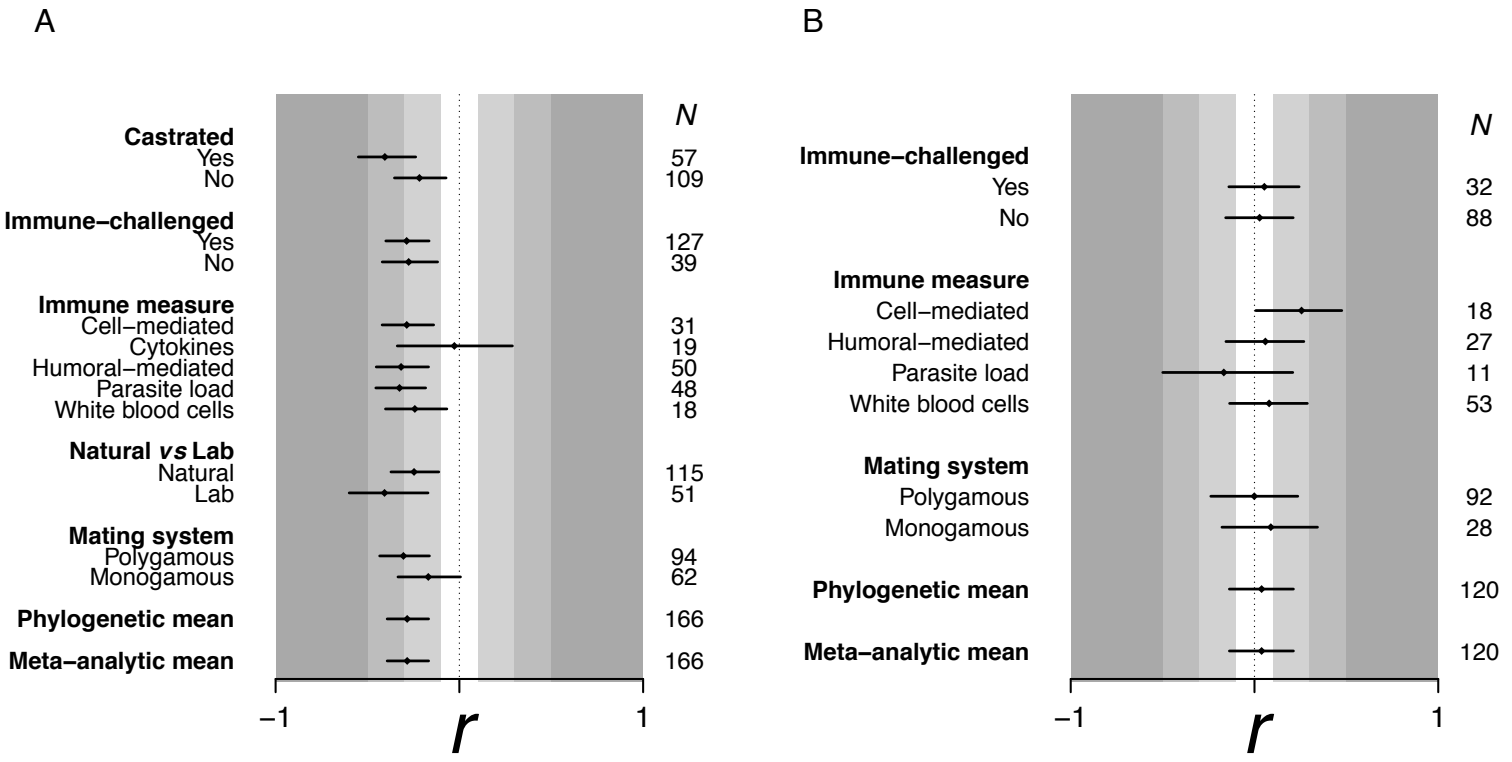

Fig. 2. Parameter estimates for (A) studies investigating the effect of testosterone manipulation on immune function and (B) studies investigating the correlation between circulating testosterone level and immune function in males. Diamonds represent the mean and error bars represent 95% confidence intervals. *N* refers to the number of effect sizes. White, light-grey, medium-grey and dark-grey spaces represent the regions for small, small-to-medium, medium-to-large, and large effect sizes, respectively, based on Cohen (1988).

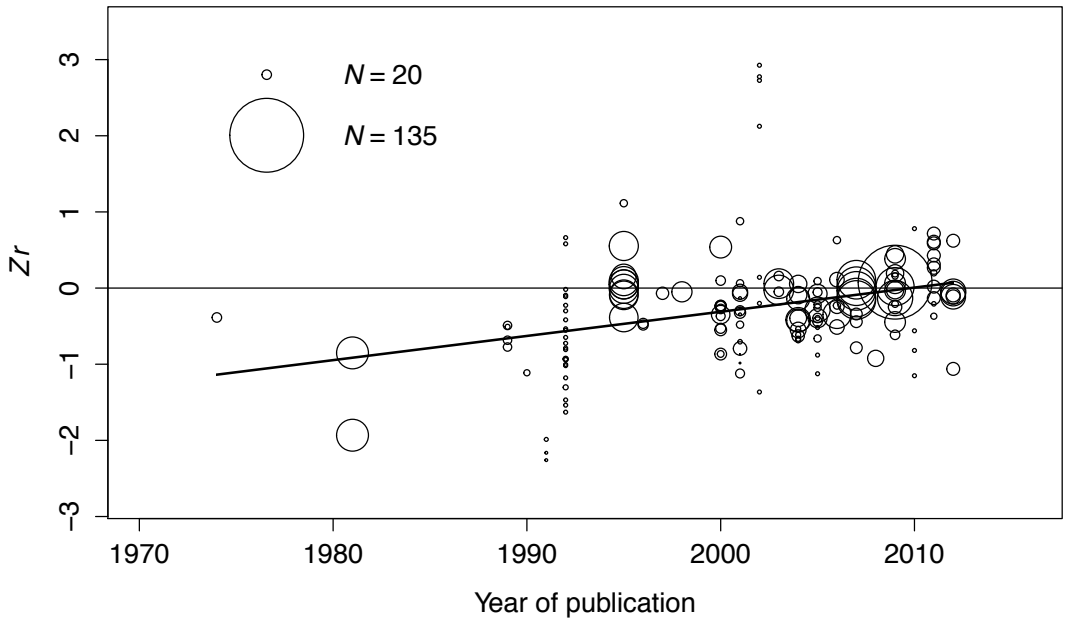

Fig. 3. Relationship between effect size and year of publication (indicated by the bold line) for studies investigating the effect of testosterone manipulation on immune function. Size of each point indicates the sample size of that effect size.

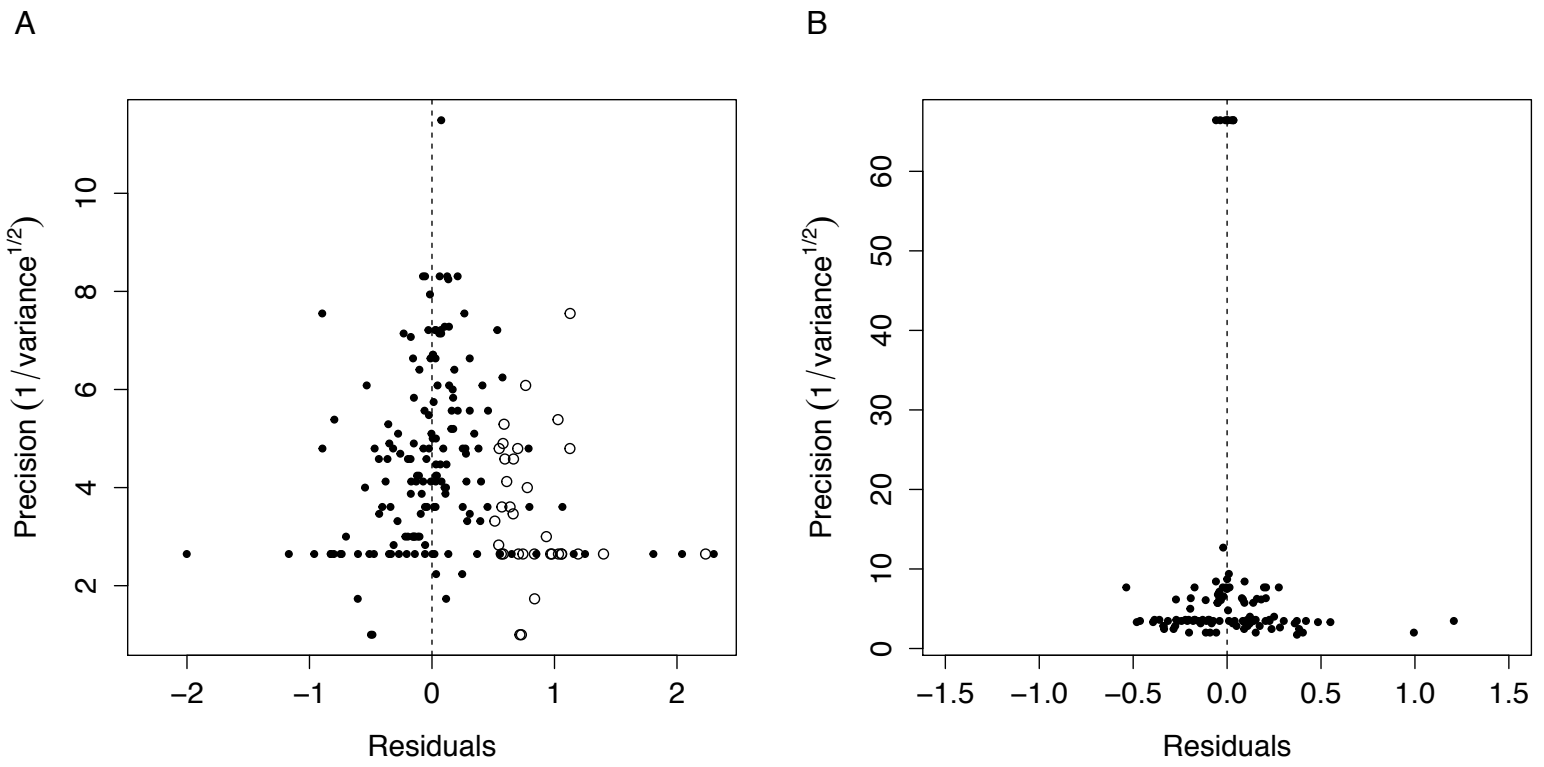

Fig. 4. Funnel plot of the residuals plotted against precision for (A) studies investigating the effect of testosterone manipulation on immune function in males and (B) studies investigating the correlation between circulating testosterone level and immune function in males. Filled circles are actual effect sizes and empty circles are missing effect sizes estimated from the trim-and-fill analyses. Dashed lines indicate the zero line.

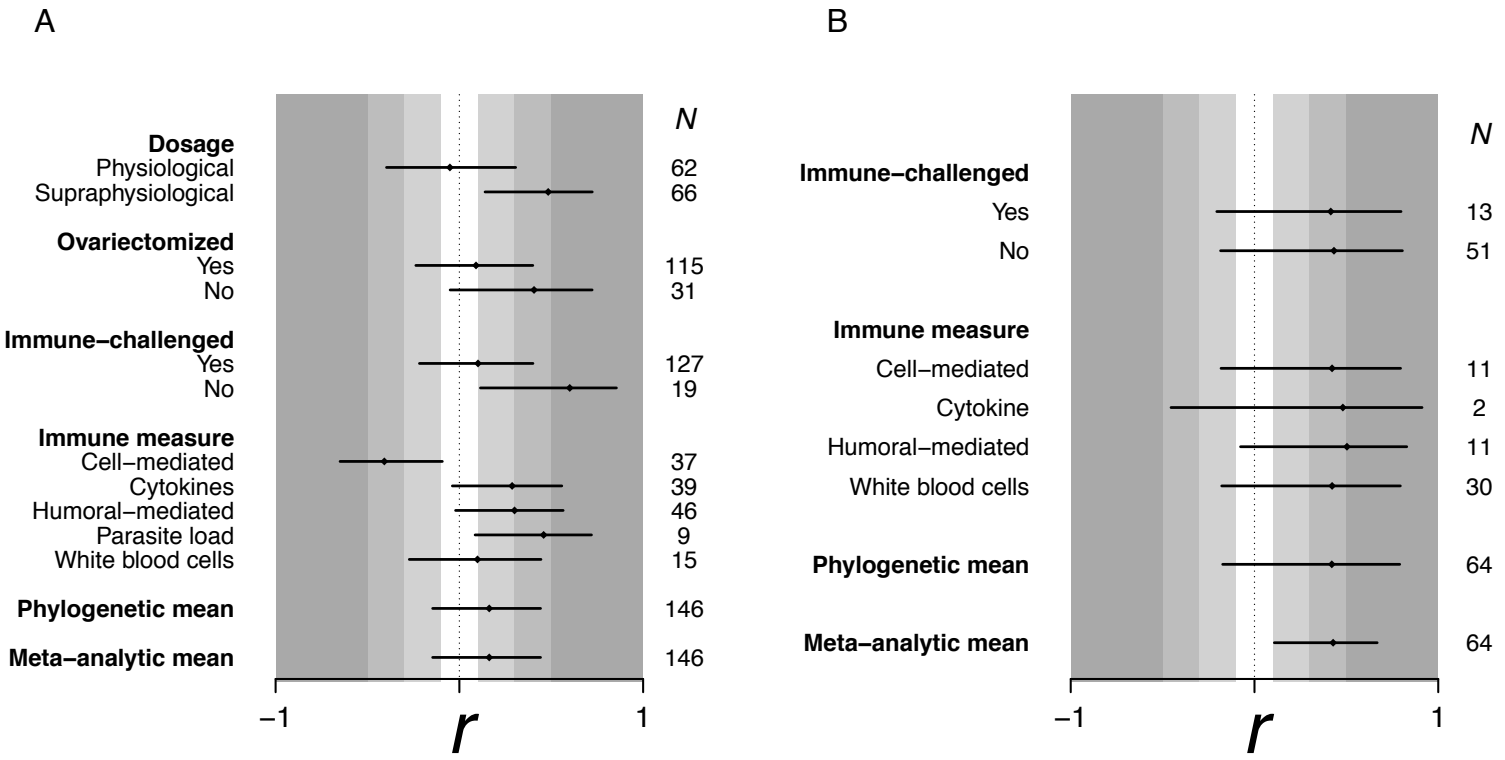

Fig. 5. Parameter estimates for (A) studies investigating the effect of oestrogen manipulation on immune function and (B) studies investigating the correlation between circulating oestrogen level and immune function in females. Diamonds represent the mean and error bars represent 95% confidence intervals. *N* refers to the number of effect sizes. White, light-grey, medium-grey and dark-grey spaces represent the regions for small, small-to-medium, medium-to-large, and large effect sizes, respectively, based on Cohen (1988).

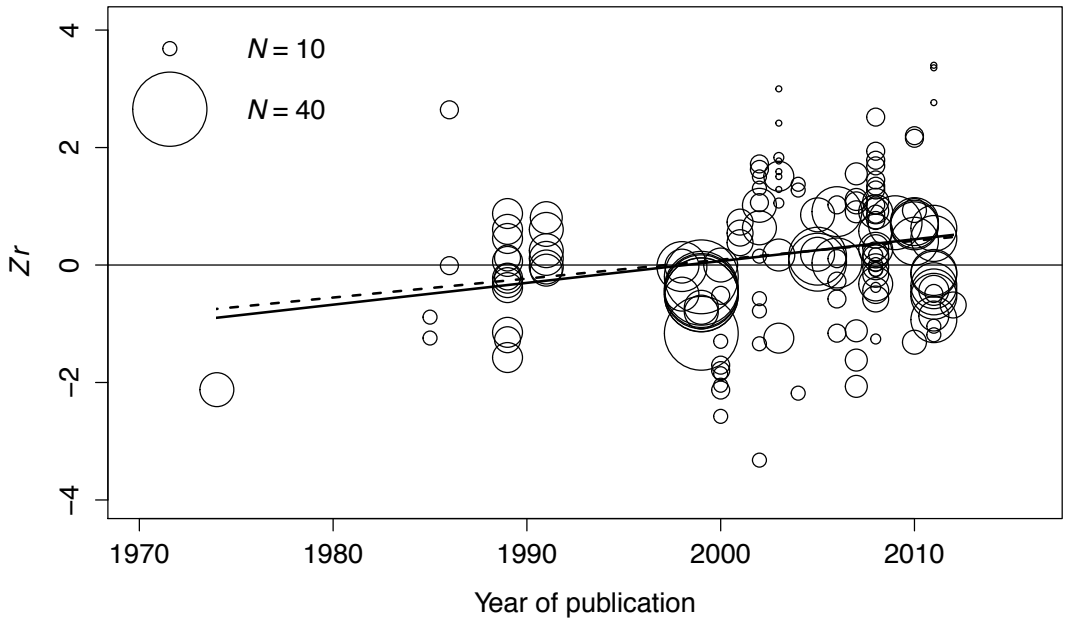

Fig. 6. Relationship between effect size and year of publication for studies investigating the effect of oestrogen manipulation on immune function. Solid line indicates the relationship when all effect sizes were included. Dashed line indicates the non-significant relationship after removing the effect size from 1974 that appeared to be driving the significant relationship between effect size and year of publication. Size of each point indicates the sample size of that effect size.

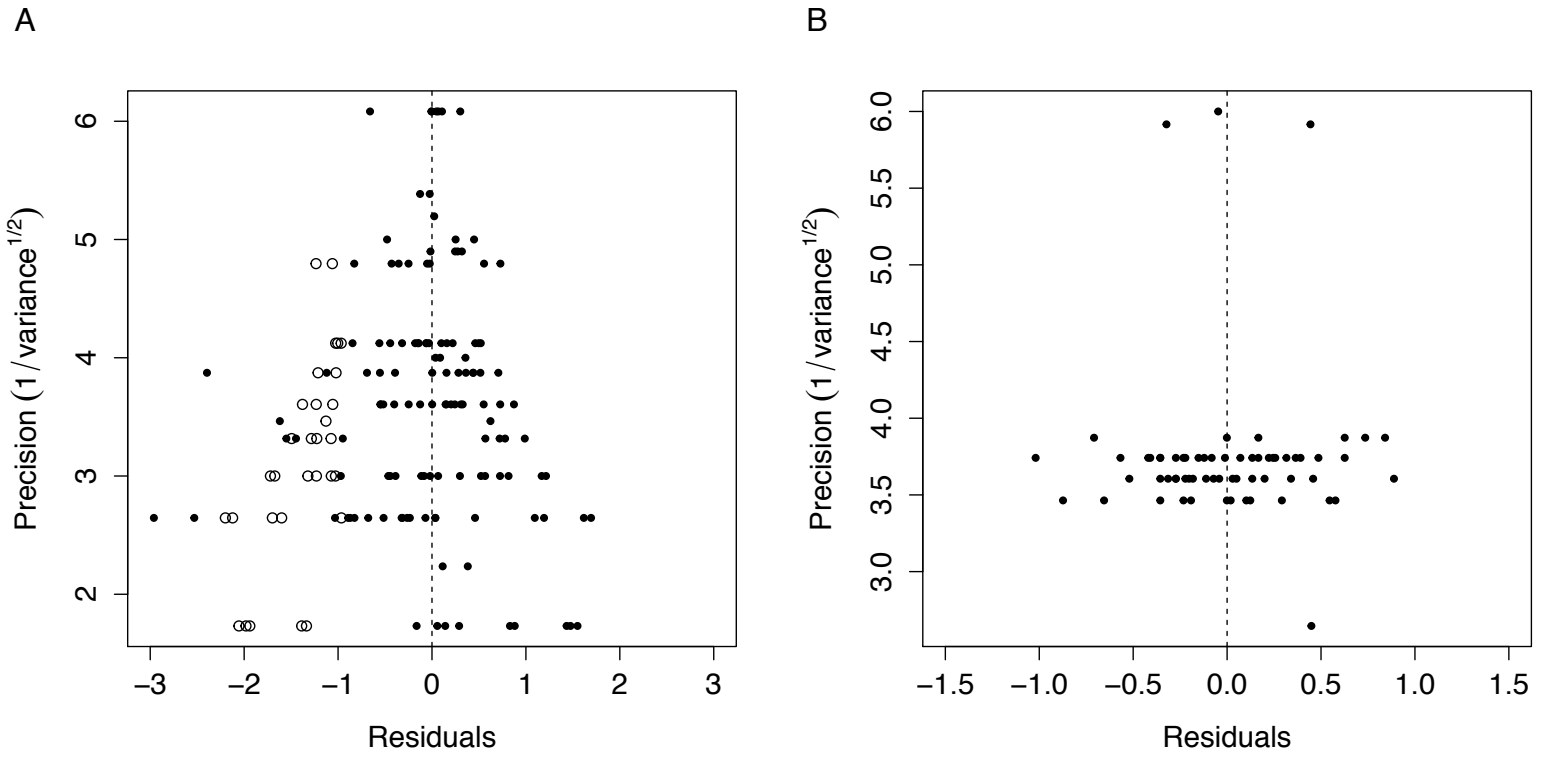

Fig. 7. Funnel plot of the residuals plotted against precision for (A) studies investigating the effect of oestrogen manipulation on immune function in females and (B) studies investigating the correlation between circulating oestrogen level and immune function in females. Filled circles are actual effect sizes and empty circles are missing effect sizes estimated from the trim-and-fill analyses. Dashed lines indicate the zero line.