Netherlands Organisation for Scientific Research

Vernieuwingsimpuls / Innovational Research
Grant application form 2017 **Vidi scheme**

Registration form (basic details)

1a. Details of applicant

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1b. Title of research proposal

Transitions in male-female differentiation: unravelling the genetic architecture and evolution of sexually dimorphic trait variation in parasitoid wasps.

1c. Summary of research proposal (299 words)

In the animal kingdom the difference between male and female appearance can range from striking to inconspicuous. Insects are well-known for their eye-catching colour differences or extreme size differences between the sexes. These sexually dimorphic traits are subjected to strong evolutionary forces and diversify rapidly within closely related genera or even within a genus, ultimately driving speciation. It was expected that the origin of new morphological traits would be caused by the appearance of new genes.

Recently, a variety of studies show that most evolutionary novelties are caused by changes in the regulatory elements of pre-existing genes, so called ***cis*-regulatory elements**. *Cis*-regulatory elements contain binding sites for transcription factors to regulate gene expression. One essential transcription factor for sexual differentiation in insects is ***doublesex***, which is involved in sex- and species-specific differences of e.g. pigmentation patterns, wing size, wing colour patterns and leg bristle number. The **target genes** on which *doublesex* acts and **developmental and regulatory mechanisms** underlying the rapid evolutionary changes are **still poorly understood**.

Here, I propose to unravel **the molecular basis of sexually dimorphic trait variation** and its **evolution on three different evolutionary timescales**: between genera, within a genus and within species of parasitoid wasps. Parasitoids are insects that live in or on other insects and ultimately kill their host. I will first identify *doublesex* downstream targets and the *cis*-regulatory element that *doublesex* binds to. With this knowledge, I will then unravel the regulatory role of *doublesex* in three sexually dimorphic traits on three evolutionary timescales: leg pigmentation, sex pheromone composition and wing size within the subfamily, on genus and species level. Together, my VIDI-program will provide **novel insights into molecular mechanisms underlying speciation**, which can eventually lead to in the development of new synthetic genetics-based tools for biological control of pest insects by parasitoid wasps.

1d. Keywords (Max. five words)

Sexual dimorphism; evolution; transcription factor; *doublesex*; parasitoid wasps

1e. Current institution of employment

Wageningen University & Research, Plant Sciences Group, Laboratory of Entomology

1f. Prospective host institution (If known)

Wageningen University & Research, Plant Sciences Group, Laboratory of Entomology

1g. NWO domain (Choose one)

Applied and Engineering Sciences (TTW)	
NWO Science Domain (ENW)	X
Health Research and Development (ZonMw)	
Social Sciences and Humanities (SGW)	
Cross-domain committee (DO)	

Explanation of the cross-domain character of the proposal (Only if you have chosen to submit your application as cross-domain; fifty to one hundred words)

1h. Main field of research
If applicable: also list other fields of research, in order of relevance.

Code	**Main field of research**
22.90.00	Biology, other
	Other fields of research (if applicable)
21.40.00	Genetics
22.40.00	Ecology

Please note that **it is compulsory** to fill out **the same, identical** information in the ISAAC/ProjectNet system on the tab "General Information" (Algemeen) section "Research fields" (Disciplines) before submitting the proposal.

1i. Public summary of your research proposal

Please supply both an English and a Dutch version (max. fifty words each), including an English and a Dutch popular title. Please check the Notes for the requested format.

Hoe ontstaan de verschillen tussen mannen en vrouwen?

Dr. E.C. (Eveline) Verhulst (v), WUR – Laboratorium voor Entomologie

Mannen en vrouwen zien er vaak heel anders uit en bij insecten zijn de verschillen enorm. Hoe deze verschillende kenmerken ontstaan is nog erg onduidelijk. Komen er nieuwe genen bij of veranderen genen van structuur? Dit project zal de regulatie van drie recent ontstane geslachts-specifieke kenmerken in insecten onderzoeken.

How do differences between males and females evolve?

Dr. E.C. (Eveline) Verhulst (f), WUR – Laboratory of Entomology

Males and females often have distinct appearances, especially insects have striking differences. The underlying mechanisms by which the different characteristics emerge are still quite unclear. Do new genes appear or do genes change structure? This research will study the molecular basis of three sex-specific characteristics that have recently arisen in insects.

Research proposal

2a1 and 2a2. Description of the proposed research
(Max. 4,000 words on max. eight pages)

2a1. Overall aim and key objectives

SCIENTIFIC RELEVANCE AND CHALLENGES

Becoming male or female – Sex determination followed by differentiation is one of the most important events during development. After all, the consequence of this event, being male or female, has profound effects on the biology of the individual, its role in the ecosystem and its life-history. Therefore, **sexual traits are extremely important components on which evolutionary forces can act to drive speciation**.

Sexual selection causes speciation – The central issue in speciation studies is to understand the arise of initial gene flow barriers between populations[1]. **Sexual selection** is an important driver of gene flow reduction as offspring production depends on mating opportunities[2]. If a trait gives the individual an advantage in securing a mate it is subject to sexual selection[3,4], e.g. deer antlers for male-male competition or the peacocks ornamental train for influencing female choice. Sexual trait evolution is also driven by female preference for males in the best condition which often correlates to trait expression[5–7]. This can end-up in a runaway process in which female preference leads to exaggeration of male traits[8,9]. Accordingly, sexual selection creates diverse sexually dimorphic phenotypes by **rapid evolutionary shifts** in reproductive behaviour, morphology and physiology. The differences in male and female appearance and physiological structures are termed **sexual dimorphism**, and ranges from inconspicuous differences to visually striking characteristics. Especially insects display a huge variety of sexually dimorphic traits including size, colorations, markings, and ornamentation. Therefore, insects are ideal subjects to study **the genetic architecture and evolution of sexually dimorphic trait variation within and between species**.

Sexually dimorphic traits evolve rapidly – Within insects, many rapidly evolving sexually dimorphic traits are known. So far, the genetic mechanisms underlying trait variation have only been studied for a few traits in the model insect *Drosophila*[10,11]. For example, male flies of the Sophophora subgenus recently acquired a set of front leg bristles, named sex combs, which are important for male mating success. The presence and tooth number of the bristles vary dramatically between species[12,13] and this is linked to rapid changes in the regulatory interaction of the body plan gene *Sex combs reduced* and the sexual differentiation gene *doublesex*[14,15]. Male-specific posterior abdominal pigmentation is also recently acquired in the *D. melanogaster* lineage, and is controlled by the interaction of the regulatory region of the pigmentation gene *bric-à-brac* with body-plan gene *Abdominal-B* and *doublesex*[16–18]. In addition, wing pigmentation patterns[19] and thorax patterning[19–22] are also examples of highly variable phenotypes within and between species and appear to have contributed to the species divergence in the *Drosophila* genus. Yet, **the developmental and regulatory genetics of sexually dimorphic traits remain poorly understood** although many species-specific sexually dimorphic traits are known.

The origin of new morphological traits may suggest the formation of new developmental pathways; however, as the above examples already indicate, a variety of studies now show that most **evolutionary novelties** are not caused by the appearance of new genes, but **are rather caused by changes in the regulatory elements of pre-existing genes, so called *cis*-regulatory elements**[23]. *Cis*-regulatory elements

contain binding sites for one or more transcription factors to regulate gene transcription. A single transcription factor may bind to many *cis*-regulatory elements; thus transcription factors usually have a pleiotrophic effect. These pleiotrophic effects can put a constraint on transcription factor evolution forcing the evolution of sexually dimorphic traits mainly through variation in *cis*-regulatory element sequence and frequency[24].

***Doublesex* controls all sexual differentiation** – The essential transcription factor for sexual differentiation in insects is *doublesex* (*dsx*)[25,26], a highly conserved gene at the bottom of the insect sex determination cascade[27–29]. It is spliced into sex-specific variants that translate into female- (DSX-F) and male-specific (DSX-M) protein isoforms. Both DSX-F and DSX-M share a domain that is also identified in mammals and other vertebrates[25,26].

In all studied insect species, **DSX is involved in sex- and species-specific differences**[30–33] predominantly by binding to *cis*-regulatory elements of target genes[34], but *dsx* can also be a transcriptional target itself by regulatory interactions with body-plan genes[15,18,33,35,36]. Despite recent research, DSX target genes and binding sites are still largely unknown and the enormous variety and sometimes extreme differences between male and female phenotypes beg for further study of these genes and their regulation. **We understand so little of these developmental pathways that are so conserved, but nevertheless result in so much biodiversity.**

My VIDI proposal: Gain more insight into the rapid evolution of sexually dimorphic traits – In my research I aim to understand **the evolutionary forces underlying speciation using highly innovative approaches to study sex determination and differentiation at the molecular level**. I primarily use the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) that has a suite of sexually dimorphic traits that vary not only between sexes but also between species (Fig. 1). One notable feature is wing size which is only sexually dimorphic in *N. vitripennis.* Males have short wings and cannot fly, while females have long wings and do fly[37]. All three other male and female species in the genus have long wings and flight capabilities[37,38]. In addition, leg pigmentation is a sexually dimorphic trait in all four *Nasonia* species; females have dark brown pigmented antennae and legs, while males lack pigmentation and have bright yellow antennae and legs[37,38]. Lastly, all four *Nasonia* species have their own blend of sex pheromones that play a role in mate recognition and sexual communication which both evolve and diversify rapidly[39,40]. Male *Nasonia* attract virgin females of the same species by releasing long-range sex pheromones that differ in number and quantity of the components, with *N. vitripennis* males releasing an additional component[41–43]. Both sexes produce long-chain saturated and unsaturated polar waxes called cuticular hydrocarbons (CHC) originally to prevent desiccation[44,45]. Female CHCs function also as short-range pheromones for males in most *Nasonia* species[40,46]. Males and females show unique, non-overlapping CHC profiles in all four *Nasonia* species; the *N. giraulti* female CHC profile diverged most from the other species and is no longer perceived by *N. giraulti* males[40]. Candidate genes for long-range sex

Figure 1: *N. vitripennis* male (top) and female (bottom). Arrows indicate the sexually dimorphic traits, dotted arrow for male morphology, solid arrow for female morphology. Colors match the objectives; in orange pheromone composition, in blue leg pigmentation, in green wing size.

pheromones[47] as well as for CHC synthesis[48] were recently identified for *Nasonia*.

Previously, I elucidated the sex determination genes[49–51] and demonstrated how these genes interact in *N. vitripennis*[51–54], thereby resolving the long standing question of its sex determination mechanism. This included identifying *doublesex*[49] and its role in the development of sex- and species-specific wing-size differences[32].

In my **NWO-VENI project** I studied *Nasonia dsx* and the evolutionary conservation of *dsx* in sexual and asexual parasitoids. I started with investigating the role of *dsx* as master sex switch[25]. Currently, I am finishing the functional characterization of *N. vitripennis dsx* splice-variants(Verhulst *et al.* in prep). I also characterized an additional *N. vitripennis* sex determination gene[55] and I collaborated on the identification of the sex determining mechanism of the parasitoid wasp *Asobara tabida*[56].

With my VIDI proposal I aim to **understand the molecular basis of sexually dimorphic trait variation and its evolution on three different evolutionary timescales: between five closely related parasitoid genera, within a parasitoid genus and within species.** To do this, I first need to know the genetic architecture of a DSX binding site. Based on research in *Drosophila* that is extended to Diptera[57], I expect that the DSX binding site consensus sequence is highly conserved within the Hymenoptera and can be identified in other parasitoid wasps once identified in one species.

KEY OBJECTIVES

(Figure 2)

1) Identify regulatory elements and target genes involved in sexual differentiation in *N. vitripennis* by combining a **genome-wide transcription factor binding site survey** with **transcriptome-wide expression analysis**.
2) Understand the gain of the sex-specific regulation of leg **pigmentation** within the Pteromalinae subfamily by **characterizing DSX regulatory elements** and link this to changes in **target gene expression** across genera.
3) Identify the role of *dsx* in the regulation of sex-specific **pheromone** differences in the *Nasonia* genus by **pheromone composition analysis** and **behavioural assays** after *dsx* RNAi, and **link this to regulatory elements in the upstream** region of target genes.
4) Elucidate the role of *dsx* in sex specific **wing-size** dimorphism in *N. vitripennis* by **splice-variant expression analysis** and **RNAi assays** comparing males and females.

ORIGINALITY AND INNOVATIVE CHARACTER

The proposed project is pioneering for several reasons:

- **First time identification of *dsx* regulatory elements in non-model species.** I will use the highly novel method of DNA affinity purification sequencing (DAP-seq)[58] to identify the downstream targets with their regulatory elements of the four *N. vitripennis dsx* isoforms. This research will be the first to use this novel technique to identify *dsx* binding sites that have never been directly identified outside of *Drosophila*[57]. Only in horned beetles have recently *dsx* target genes and binding sites been indirectly identified using transcriptome-wide analysis after systemic knockdown[33]. I will combine the best of both worlds and use DAP-seq and transcriptome-wide expression analysis following *dsx* RNAi knockdown to yield highly robust data that is useful to extract general principles on *dsx* transcription factor

binding. This will simplify the identification of *dsx* target genes in Hymenoptera and open-up the field of regulatory evolution.

- **Unique approach to study the regulation of pheromone composition.** I will study the role of DSX in the production of sex- and species specific long-range pheromones and short-range cuticular hydrocarbons. The role of sex pheromones in mate recognition and courtship has been studied extensively in *Nasonia* but the genetic mechanism underlying sex-specific regulation have not been studied before.
- **Novel study of DSX regulatory evolution across different evolutionary time scales.** I will identify DSX binding site sequence variation and frequency in the pigmentation genes across species by comparing five sister species from different parasitoid genera. As DSX binding sites have not been identified in Hymenoptera before, and their evolution has only been studied in *Drosophila* species[34,57], this research will yield new insight into the evolution of a newly acquired sexually dimorphic trait.
- **New insights into role of DSX in regulating sexually dimorphic traits.** I will investigate in what way *dsx* regulation is an integral part of the regulatory pathway in establishing sexually dimorphic phenotypes. In *Nasonia* we have shown that species-specific male wing size variation is linked to the species-specific *cis*-regulatory element of *dsx* itself, indicating that regulatory control of *dsx* is required for this dimorphic trait as well[32]. However details of the transcription factor targeting *dsx* are unknown, it may very well be that *dsx* regulates its own expression. This research will be among the first to uncover what the role is of *dsx* expression levels in regulating dimorphic trait development.

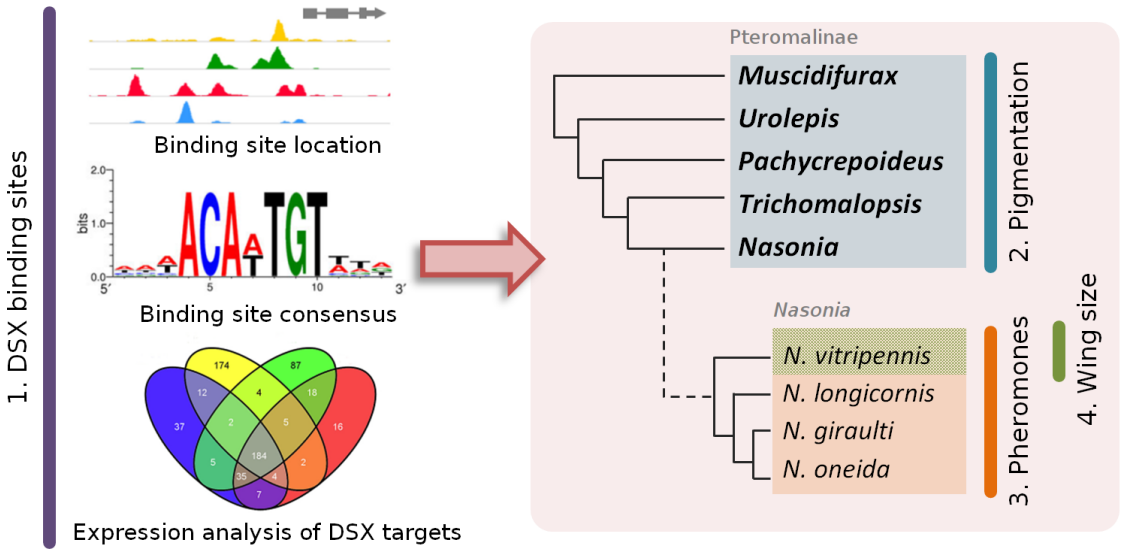

Figure 2: Overview of the proposed research objectives and the phylogenetic tree with the study species. Blue square: the Pteromalinae subfamily; orange square: *Nasonia* species.

METHODS AND TECHNIQUES

Study system

The main system that will be used in this proposal is *Nasonia*, a genus of four closely related wasp species belonging to the subfamily Pteromalinae (Hymenoptera: Pteromalidae). The *Nasonia* genus diverged 1 million year ago from its closest relative, *Trichomalopsis sarcophagae*[38], and all four *Nasonia* species are interfertile when cured from the endosymbiont *Wolbachia*[59]. Especially *Nasonia vitripennis*, has been the subject

of ecological, evolutionary and developmental genetics for over 50 years and has become a model species for all parasitoids[60]. Its genome is sequenced and fully annotated[50] and their short generation time, easy handling and still expanding array of genomic, transcriptomic and functional resources[61], such as (parental)RNAi[62], CRISPR/Cas9 mutagenesis[63] and *in situ* hybridizations[61], makes them particularly suited for evolutionary and molecular biology research. *N. vitripennis* as the most basal species is a generalist that parasitizes many fly species, and occurs worldwide. The other three species are specialists, parasitize blowflies in bird-nests, and are endemic to North America with *N. longicornis* occurring in the west and *N. giraulti* and *N. oneida* occurring sympatrically in the east.

In addition, five species from the subfamily Pteromalinae will be used to study the molecular regulatory evolution of leg pigmentation: *N. vitripennis*, its close relative *T. sarcophagae*, *Pachycrepoideus vindemmiae*, *Urolepis rufipes* and *Muscidifurax raptorellus*. Except for the more basal species *M. raptorellus*, all these parasitoids have sexually dimorphic leg pigmentation, with females having brown pigmented legs, while males lack pigmentation and have bright yellow legs[(pers. observ.)]. In *M. raptorellus* both males and females have brown pigmented legs suggesting that this is the ancestral mode[(pers. observ.)]. Pteromalid wasps are parasitoids which live on or in other insects, thereby eventually killing their host[64]. As such, they are used extensively as biological control agents. Like all Hymenoptera they have haplodiploid sex determination in which haploid males develop from unfertilized eggs and diploid females develop from fertilized eggs.

Objective 1: Identify DSX binding sites using DAP-seq and RNA-seq

To identify the binding sites within the *cis*-regulatory elements for all four *N. vitripennis* DSX isoforms my team will use **DNA affinity purification sequencing (DAP-seq)**. It works with an affinity-purified transcription factor followed by next-generation sequencing of the binding site enriched genomic DNA library[58]. This was recently successfully used to identify the *Arabidopsis* cistrome[65]. The identification and location of the DSX binding sites will also reveal the, mostly unknown, DSX downstream target genes. The results from the DAP-seq assay will be **verified** by *dsx* knockdown using **RNA interference (RNAi)** followed by transcriptome-wide **RNA sequencing (RNA-seq)**. Genes that are under DSX regulation will show differential expression compared to the control. The results of the DAP-seq will be compared with the identified target genes after RNAi and RNAseq to 1) **verify** that some differentially expressed target genes after *dsx* knockdown contain one or more DSX binding sites in their regulatory region; 2) **distinguish** primary from secondary DSX targets, as the latter are not expected to contain DSX binding sites in their promoter region; 3) **provide** robust data.

1a. DAP-seq with four DSX isoforms – Affinity-tagged *N. vitripennis* DSX isoforms DSX-M1, DSX-M2, DSX-F and DSX-U will be produced *in vitro* using the TNT® T7 Insect Cell Extract System (Promega) and used following the DAP-seq protocol[58]. Genomic DNA will be extracted from adult male and female wasps and mixed with the purified DSX isoforms to bind DNA. After removing unbound DNA, the enriched DNA library will be sequenced on an Illumina HiSeq, 100bp paired-end to obtain 15 million reads per DAP-seq sample. The reads will be aligned against the genome using Bowtie2[66] and peak calling analysis and motif discovery will be done using HOMER[64]. In the end we will have the core consensus DSX binding sequence and a list of genes containing one or more of DSX binding site(s) in their *cis*-regulatory element(s).

1b. *dsx* RNA-seq – An RNAi assay targeting all *dsx* splice variants simultaneously will be used to knock down *dsx* in 4th instar larvae in *N. vitripennis*, *N. giraulti*, *N. oneida* and *N. longicornis* using microinjections. The resulting adults will be sexed and three different tissues will be collected in triplicate per sex for RNA extraction: thorax including wings

and legs, abdomen, and head. RNA samples will be sequenced on an Illumina HiSeq, 150bp pair-end 20 million reads per sample. Paired end sequencing in triplicate allows for enough statistical power to do isoform expression analysis[67]. The control samples will be treated with an exogenous target, *gfp*, to control for off-target effects due to RNAi itself[68,69]. In total 144 RNAseq samples will be sequenced (3 tissues, 4 species, 2 sexes, 2 treatments, in triplicate). Differential gene and isoform expression for all sets will be analyzed using the Tuxedo pipeline[70]. The genes that are differentially expressed in the *dsx* RNAi sample are considered DSX target gene candidates; however they could be directly regulated by DSX or via multiple intermediate proteins.

Objective 2: DSX regulation of leg pigmentation in Pteromalinae

I have found that *dsx* RNAi silencing in *Nasonia* pre-pupal stage results in female-like leg pigmentation in males, while leaving females unaffected. This suggests that DSX-M represses leg pigmentation in males(Verhulst, unpublished data). As male leg pigmentation appears to be the ancestral state, I expect that the gene responsible for leg pigmentation acquired one or more DSX binding sites in its *cis*-regulatory element to bind DSX-M. The gene underlying pigmentation is suggested to be *yellow*, which is responsible for black pigment formation in *Drosophila*[71]. In *Nasonia* we expect to identify this gene and its *cis*-regulatory region with the DSX binding site in objective 1b. The DSX binding site consensus sequence will be identified in objective 1a.

2a. Identify DSX binding sites in pigmentation gene – When the exact structure and sequence of the pigmentation gene with its cis-regulatory element are identified in objective 1, these regions will be aligned against the published genome of *T. sarcophagae* (NCBI: NNAY00000000)[72]. No genome sequences have been published for the other three species. Therefore, a conserved region within the putative pigmentation gene will be identified for *M. raptorellus*, *P. vindemmiae* and *U. rufipes* from which a genome walk into the *cis*-regulatory region can be made[73] using the Universal GenomeWalker™ 2.0 kit (Clonetech). After the *cis*-regulatory region of the putative pigmentation gene is identified, comparative analysis will indicate sequence differences between the species which can be linked to the presence or absence of dimorphic leg pigmentation.

2b. qRT-PCR of pigmentation gene in legs – To test whether expression of the putative pigmentation gene is correlated to the presence and number of DSX binding sites we will measure the expression levels of the putative pigmentation gene in adult male and female legs for all five species by quantitative-reverse transcriptase polymerase chain reaction (qRT-PCR) and relate this to the commonly used housekeeping gene *elongation factor 1 alpha*.

Objective 3: DSX regulation of sex pheromone synthesis in *Nasonia*

Preliminary experiments of *N. vitripennis* mating behavior after *dsx* knockdown indicated that treated males are recognized as females, possibly because their CHC profiles are shifted from male-specific to female-specific(Verhulst, unpublished data). This could indicate that normally *dsx* directly regulates pheromone biosynthesis genes to produce a male-specific CHC profile. In addition, females did not respond to *dsx*-knockdown-male presence suggesting that the long-range pheromones are not produced or not perceived(Verhulst, unpublished data).

3a. Larval *dsx* RNAi in all four species and both sexes – *Dsx* will be knocked down as described in objective 1b after which the adults will be used for 3b and 3c. Knockdown levels will be quantified using qRT-PCR.

3b. Sex pheromone composition – After *dsx* RNAi, the composition of the long-range male sex pheromones and male and female CHC profile will be determined by extraction of the pheromones and analysis on a gas chromatography/mass spectrometry (GC/MS)

according to Ruther *et al.*[41] for long-range pheromone and Büllesbach *et al.*[40] for the CHC.

3c. Behavioral assays – *Nasonia* displays distinctive courtship behaviors that includes several stages and is well described[74–76]. Behavioral assays will be performed according to Diao *et al.*[47]. *Dsx* RNAi treated and *gfp* RNAi treated (control) virgin individuals will be pairwise compared to determine the effect of *dsx* knockdown on the courtship and mating behavior of males and females.

3d. Identification and comparison of DSX binding sites – The consensus DSX binding site identified in objective 1a is aligned against the candidate genes involved in the production of long-range pheromones[47] and CHCs[48] to determine the presence and number of binding sites.

Objective 4: DSX regulation of wing size dimorphism in *Nasonia vitripennis*

By backcrossing the *N. giraulti* wing size locus *ws1* into a *N. vitripennis* genetic background it was possible to obtain genetic *N. vitripennis* males with 45% longer wings[32]. I collaborated on tracing the *ws1* region to the 5' non-coding cis-regulatory region of *dsx* and it shows a number of sequence differences between *N. vitripennis* and *N. giraulti*. qRT-PCR showed that *dsx-M* is higher expressed in *N. vitripennis* developing wings compared to *N. giraulti*[32]. In addition, another identified candidate gene (*unpaired-like*) is involved in species-specific male wing width and contains multiple non-coding sequences contributing to the wing size differences between species[77]. In both cases it is unknown what factor binds to the regulatory sites.

4a. qRT-PCR of *dsx* splice variants in wing tissue – *Dsx* isoform expression will be determined in developing wing tissues that are dissected from 4th instar larvae[32] using the latest *dsx* isoform information(Verhulst *et al.* in prep).

4b. *dsx* RNAi in both sexes with sex-specific isoforms – To determine the role of the DSX isoforms in wing development, all four *dsx* isoforms will be silenced in 2nd instar larvae produced by virgin and mated females. Virgin females will only produce males; mated females will produce nearly 90% females. Both *dsx* knockdown levels and the *unpaired-like* expression levels will be quantified by qRT-PCR

4c. Quantification of wing size abnormalities – After *dsx* RNAi, the wings of males and females will be measured according to Loehlin *et al.*[32,77] to determine the effect of the different DSX isoforms on wing length and width.

4d. Binding site analysis and mutagenesis with CRISPR/Cas9 – The binding sites consensus from objective 1 will be aligned to the regulatory region of *dsx* and *unpaired-like* to determine its presence and frequency. If present, CRISPR/Cas9 mutagenesis will be used[63] to change the regulatory regions of both genes to determine the effect on wing size and width. In any case CRISPR/Cas9 mutagenesis will be used to change step-by-step the regulatory region of *N. vitripennis dsx* to that of *N. giraulti* based on the polymorphisms found in Loehlin *et al.*[32] and determine the effect on wing development.

2a2. Research plan:

WORKPLAN AND TIME TABLE

The project will be conducted by two PhD students (1.0 fte – 4 years), a postdoc (0.8 fte – 3.5 years), and myself (0.5 fte – 5 years). The post-doc will identify the *dsx* binding sites and spend most of its time analysing the DAP-seq and RNA-seq data for which a strong bioinformatics background is required. PhD1 will focus on the sex pheromones, and will collaborate with the postdoc to meet objective 3d. PhD2 will focus on the wing size and will collaborate with the postdoc to design the targets for CRISPR/Cas9 mutagenesis in objective 4d. PhD1 and PhD2 will both collaborate on the pigmentation analysis, with PhD1 focusing on the genomic walk to get the binding sites in the five

species and PhD2 focusing on the expression analysis of the pigmentation gene, PhD1 will collaborate with the postdoc to obtain the pigmentation candidate. The applicant is responsible for project coordination and synthesis of the results as well as training PhD1 and PhD2.

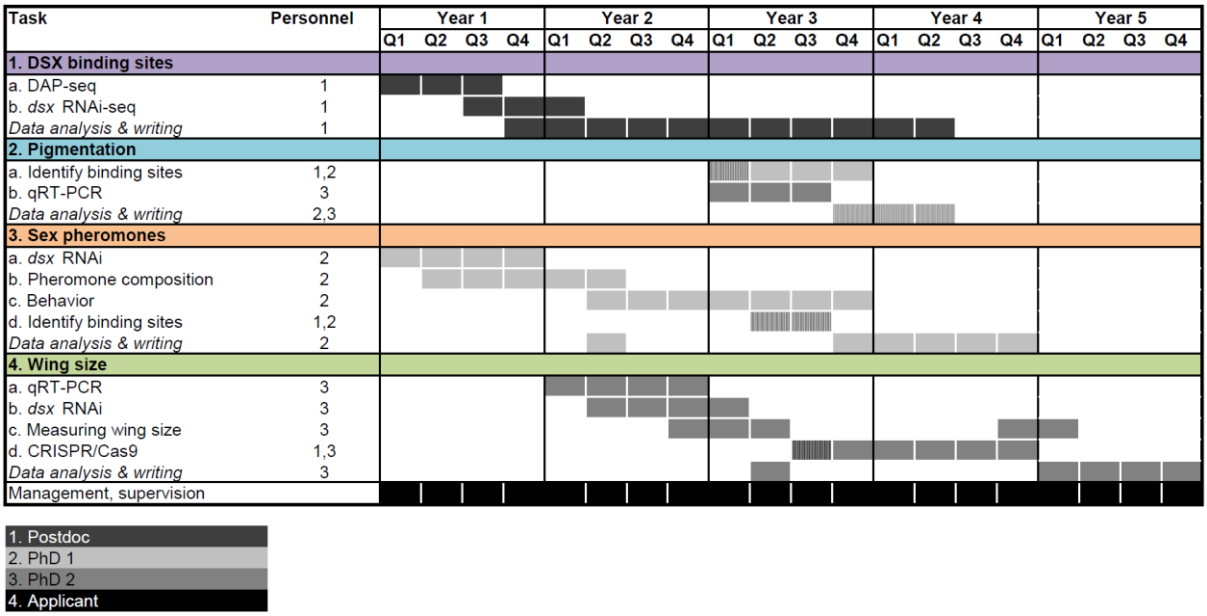

Figure 3: Gantt chart outlining experiment planning in project years. The applicant is involved in management, supervision, data analysis and writing of all tasks.

LOCAL, NATIONAL AND INTERNATIONAL COLLABORATION

Local – The research is hosted at the Laboratory of Entomology at Wageningen University where I collaborate with Dr. Hans Smid on the use of microscopy and parasitoid microinjections needed for RNAi. Furthermore, there is chemical ecology expertise in the group (Alexandre Villela, Prof. Marcel Dicke). In addition, I have collaborations with Dr. Bart Pannebakker, WUR Genetics, an expert on *Nasonia* life history evolution and population genetics.

National – I collaborate extensively with Prof. Leo Beukeboom, Dr. Louis van de Zande and Dr. Elzemiek Geuverink at Evolutionary Genetics (University of Groningen) on the molecular mechanisms of sex determination in parasitoid wasps and the application of CRISPR/Cas9 in *Nasonia*. I will initiate collaborations with Prof. Jacintha Ellers (Vrije Universiteit Amsterdam), an expert on gene-environment interaction and trait diversity in insects.

International – I have several international collaborations relevant for this project; I collaborate with Dr. Carol Trent on the *doublesex* variants in *Nasonia* (Western Washington University, USA), I will continue to collaborate with Dr. Jeremy Lynch on *Nasonia* embryonic development (University of Chicago, USA), with Dr. Daniel Bopp on sex determination (University of Zurich, Switzerland). The project will benefit enormously from my collaboration with Prof. John (Jack) Werren on *Nasonia* wing size and *Nasonia* genomics (University of Rochester, USA) and he will provide me with the wing-size backcross strains. I will initiate collaboration with Prof. Joachim Ruther (University of Regensburg, Germany), an expert on long-range sex pheromones and cuticular hydrocarbons, and I will continue the recently started collaboration with Prof. Jürgen Gadau (University of Münster, Germany) on *N. vitripennis* and *N. giraulti* resequencing and cuticular hydrocarbons. Lastly, I will start collaborating with Dr. Patrick Ferree (Claremont McKenna College) on *Nasonia* embryonic microinjections.

2b. Knowledge utilisation

(Max. 1,000 words on max. two pages)

POTENTIAL

This proposal represents curiosity-driven, fundamental research—the primary aim is to improve our understanding of the molecular mechanisms and genetic architecture that allow rapid turn-over of sexually dimorphic traits. We have only scraped the tip of the iceberg when it comes to understanding how the conserved transcription factor *doublesex* can **regulate so many different sexually dimorphic traits** that vary so dramatically between species[25,26]. The outcomes of this research will greatly aid our insight into the evolutionary forces that **act on these important, conserved developmental programs** and will benefit the fields of evolutionary and developmental biology greatly. Moreover, a number of expected results are **relevant for other scientific disciplines and society**.

Diseases – Understanding the pleiotropic nature of transcription factors and the genetic architecture of their regulatory elements will help us to understand complex diseases. Many diseases are caused, in a similar way to the evolution of sexually dimorphic traits, by mutations changing gene expression and not by mutations leading to protein changes[78]. This proposal may therefore provide unique insights as it focuses on one highly conserved transcription factor that regulates a suite of visible and invisible traits[25,26] that will deepen our insight of the genetic architecture of gene expression variation. Ultimately, understanding the evolutionary constraints that shape the *cis*-regulatory elements of genes will aid in modelling the consequences of new mutations and in **precision medicine of interest to consortia such as LifeLines**[79].

Insect control – Molecular understanding of sex determination mechanisms with its sex-specific-splicing has already been successfully used to control a number of insect pests[80–85]. Oxitec uses a method called `Release of Insects with Dominant Lethality´ (RIDL) which uses insects carrying a sex-specific conditional dominant-lethal gene[86–88] that when expressed in the wild is deadly. My proposal will add to this by 1) extending our understanding of sexual differentiation which has **relevance for biocontrol stakeholders (Koppert and Insect Pest Control Laboratory of Joint FAO/IAEA)**; 2) producing knowledge of DSX *cis*-regulatory elements, their effect on targets and the methodology to obtain these. The latter will give **researchers at e.g. Oxitec a better tool to create genetic strains**, now based on the more precise sex-specific regulation of gene expression. The incorporation of DSX *cis*-regulatory elements to sex-specifically regulate the conditional lethal has been shown for *Drosophila melanogaster* only[89,90]. Not much progress has been made for other insects as knowledge on DSX targets and binding sites is lacking. In this proposal I will use the very recently published DAP-seq technique[58] to determine transcription factor binding sites, in this case DSX, in non-model systems for which antibody based ChIP-sequencing is time-consuming and expensive. This may give non-model insect researchers finally the possibility of identifying binding sites that can be used to (sex-specifically) regulate any gene if combined with CRISPR/Cas9 mutagenesis[91]. Within the Hymenoptera a number of pest species are known (e.g. sawflies) and the DSX binding site knowledge obtained from this proposal may be directly utilized to create dominant-lethal carrying insects to be used in insect pest control which would **highly interest stakeholders such as Oxitec and Joint FAO/IAEA**.

I am currently involved in the EU-ITN Breeding Invertebrates for Next Generation BioControl (ITN-BINGO), and I have already frequent contact with stakeholders, such as Dr. Kostas Bourtzis (Insect Pest Control Section, Joint FAO/IAEA) and Tom Groot (Koppert Biological Systems). In addition, I have established contact with Martha Koukidou (Oxitec) during Nöthiger meetings. Therefore, I am in the optimal position to get relevant **results directly to stakeholders within 3-5 years** from now.

IMPLEMENTATION

Dissemination – I have extensive contacts with many stakeholders and I will discuss the results where relevant. In addition to scientific publications in high-impact journals, **I will communicate the research outcomes through several channels**. First of all, the results from this proposal will be presented to fellow researchers on large international meetings and smaller meetings, such as the yearly *Nasonia* meeting and the Nöthiger (European Insect Sex Determination) meeting, the latter being attended by both researchers and stakeholders. I will include the research topic into my lecture series in the WUR MSc course Molecular Aspects of BioInteractions. Third, I have presented my work at the yearly Netherlands Entomology meeting many times and will continue to do so. This yearly meeting for entomology enthusiasts —amateurs and professionals— provides a great platform to share scientific results with a general public. Also, I have a personal website (www.evelineverhulst.nl) where I give general information on parasitoid wasps, I share my publications, presentations and posters and I blog occasionally on scientific progress and problems. I intend to write press releases and publications in the popular press as soon as I obtain relevant results. Furthermore, I participate in Wetenschapper in de Klas (Scientist in the Classroom), an initiative from Wageningen University that aims to bring scientists to primary schools to inspire young children. I teach, with enormous pleasure, children about parasitoid wasps and, natural and sexual selection that shape the biodiversity we see around us.

Action plan – The results of my VIDI will be highly relevant for various stakeholders for the following reasons:

1) The **applicability** of DAP-seq to uncover *cis*-regulatory elements in a non-model (non-drosophilid) species for which antibody based ChIP-sequencing is time-consuming and expensive (<4 years)
2) The **knowledge** of *Nasonia* DSX cis-regulatory elements and their effects on downstream targets that may be extrapolated to Hymenoptera (<6 years)

Specifically, I expect that in the **third year**, the DSX binding sites and targets are identified, and I have confirmed their conservation in Hymenoptera and the applicability of DAP-seq for other insects. I will then **organize a meeting with the potential stakeholders** (including FAO/IAEA, Koppert and Oxitec) to seek active collaboration to use the knowledge for biocontrol applications which will be accompanied with a press release. In addition, I plan to build a database of sex-specific genes and their regulatory elements in **the fourth year**. First, this will be based on *Nasonia*, but with time additional insects will be added and can be used by stakeholders to design genetic tools for insect control.

2c. Number of words used

Section 2a: **3960** *(max. 4,000 words)*

Section 2b: **994** *(max. 1,000 words)*

2d. Literature references

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2e. Data management

Responsible data management is part of good research. For the collection/generation of data and the analysis of this data, timely measures need to be taken to ensure the storage and later reuse of the data. This means that prior to the start of the research project researchers must ascertain a) which data could be relevant and b) how these data could be stored so that they are accessible for reuse. After a proposal has been awarded funding, the researcher will draw up a detailed data management plan in which the researcher explains how all relevant data research data will be made findable, accessible, interoperable and reusable (FAIR).Please answer the following questions:

1. *Will data be collected or generated that are suitable for reuse?*

Yes

2. *Where will the data be stored during the research?*

The data will be stored on the Wageningen University Network drives that are daily backed up. An additional back up is made to the personal NAS of the PI.

3. *After the project has been completed, how will the data be stored for the long-term and made available for the use by third parties? To whom will the data be accessible?*

Sources of raw data we will collect and store are:
a. electronic lab journals in eLabJournal, score forms, data files.

b. laboratory data, such as qRT-PCR runs, pictures, movies.
c. sequence data from DAP-seq and RNAseq
d. sufficient metadata to interpret the raw data.

Requests for data before publication by external scientists are to be judged by the PI. All data will be kept for a period of at least 10 years.

After publication, all data will be stored in trusted external repositories. For many journals this is a requirement for publication. Sequence data will be uploaded to EMBL-EBI and NCBI and binding sites information will be made available through the proposed database. Data used for publications will be stored in the Dryad digital repository (www.datadryad.org), a curated resource that makes the data underlying scientific publications discoverable, freely reusable, and citable. Data not in a format accepted by such repositories will be made available through generic storage facilities such as KNAW DANS (http://www.dans.knaw.nl/).

4. *Which facilities (ICT, (secure) archive, refrigerators or legal expertise) do you expect will be needed for the storage of data during the research and after the research? Are these available?**

The Wageningen University ICT facility is needed for Network drives maintenance and is available during and after research. The PI her NAS will be available during and after research as well.

**ICT facilities for data storage are considered to be resources such as data storage capacity, bandwidth for data transport and calculating power for data processing.*

Curriculum vitae

4a. Personal details

Title(s), initial(s), first name, surname:	Dr., E.C., Eveline, Verhulst
Gender:	Female
Date of birth:	04/02/1982
Nationality:	Dutch

4b. Master's degree ('Doctoraal')

University/College of Higher Education:	University of Groningen
Date (dd/mm/yy):	31/03/2006
Main subject:	Molecular Biology

4c. Doctorate

University/College of Higher Education:	University of Groningen
Starting date (dd/mm/yy):	01/04/2006
Date of PhD award (dd/mm/yy):	21/01/2011 (*Cum laude*, top 5%)
Supervisor ('Promotor'):	Prof. L.W. Beukeboom
Thesis title:	"Genetic basis of sex determination in the haplodiploid wasp *Nasonia vitripennis*"

4d. Work experience since completing your PhD

Position	**Period** (date-date)	**FTE**	**Type of position** (fixed term/ permanent/ tenure track/ other)	**Institution**
Assistant professor* NWO-Veni	August 2017 - present	0.8	Tenure Track*	Wageningen University & Research, Laboratory of Entomology
Postdoctoral fellow NWO-Veni	April 2014 – July 2016	0.8	Fixed term	Wageningen University & Research, Laboratory of Genetics
Postdoctoral fellow	July 2013 – March 2014	1.0	Fixed term	Netherlands Institute of Ecology (NIOO-KNAW)
Postdoctoral fellow	January 2011 – June 2013	1.0	Fixed term	University of Groningen

* My current position is a Tenure Track. I am in the second year of an Assistant Professorship after a successful evaluation by the Recruitment Advisory Committee for a Tenure Track position at Wageningen University. The Tenure Track in Wageningen consists of 6 years Assistant Professor, 6 years of Associate Professor, after which a Full Professorship may be earned.

- April 2014 – present: Personal NWO Veni grant. Project title: "Insect sex determination: The road after *doublesex*". Supervision of 3 PhD students, 1 MSc student and 3 BSc students.
  - From August 2016 – present: at the Laboratory of Entomology, Wageningen University. *Tenure Track**.
  - From April 2014 – July 2016: at the Laboratory of Genetics, Wageningen University.
- July 2013 – March 2014: Postdoctoral fellow at Netherlands Institute of Ecology (NIOO-KNAW). Project title: "The epigenetics of great tit personality".

- January 2011 – June 2013: Postdoctoral fellow on NWO-ALW TOP grant awarded to Prof. L.W. Beukeboom. Project title: "Molecular mechanism of *Nasonia* primary signal".

Months spent since completing your PhD CV

January 2011: Doctorate

I. January 2011 – June 2013: 1.0 fte position, 90% research, 10% education
II. July 2013 – March 2014: 1.0 fte position, 100% spent on research
III. April 2014 – May 2015: 0.8 fte, 100% on research
IV. June 2015 – September 2015: maternity leave
V. October 2015 – July 2016: 0.8 fte (Ouderschapsverlof), 100% research
VI. August 2016 – October 2017: 0.8 fte (Ouderschapsverlof), 75% research, 15% education, 10% management

Calculation months of research

I. 30 months * 1 fte position * 0.9 spent on research = 27 months
30 months * 1 fte * 0.1 spent on education = 3 months
II. 9 months * 1 fte position * 1 spent on research = 9 months
III. 11 months * 0.8 fte position * 1 spent on research = 8.8 months
IV. 4 months maternity leave
V. 10 months * 0.8 fte position * 1 spent on research = 8 months
VI. 14 months * 0.8 fte position * 0.75 spent on research = 8.4
14 months * 0.8 fte position * 0.15 spent on education = 1.68
14 months * 0.8 fte position * 0.1 spent on management = 1.12

Experience	**Number of months**
Research activities	27+9+8.8+8+8.4=**61.2**
Education	3+2.24=**4.68**
Care or sick leave	**4**
Management tasks	**1.12**
Other (please specify):	

4e. Academic staff supervised

	Give names or numbers	**Please indicate/specify your role (for PhDs, mark <u>one</u> role)**		
PhDs		Promotor (formal supervisor)	Co-promotor (formal co-supervisor)	Role as (co-) supervisor
Ongoing	Kim Ferguson (WUR)		x	Weekly discussion and supervision
	Yidong Wang (WUR)		x	Daily discussion and supervision

Successfully completed	Elzemiek Geuverink (RuG)		x	Weekly discussion and supervision
Subtotal PhDs	3	0	3	
Postdocs				
	Zhang Juan (Julie)	Visiting postdoc researcher, weekly discussions for 3 months		
Subtotal postdocs	1			
Master students				
Subtotal master students	5	Primary supervisor for MSc thesis		
Other				
Subtotal other	3 1	Internship students Applied University (6-7 months); primary supervisor Technician; supervision and biweekly discussions for 3 years		

4f. Brief summary of your research over the last five years
(Max. 250 words)

In the past five years, I have expanded my horizon and developed my own research line on the evolution of sex determination and differentiation at the molecular level in parasitoids. My research integrates evolutionary, behavioral, developmental and morphological disciplines and aims to understand underlying molecular and genetic mechanisms. I have demonstrated the ability to generate and execute my own ideas and have become an independent scientist, handling contrasting projects successfully. Moreover, I am capable of supervising early-career postdocs, PhDs, MSc and BSc students and technicians.

Since 2011, I have identified the molecular mechanism of the primary sex determination signal in *N. vitripennis* on which I still collaborate with Evolutionary Genetics at University of Groningen. Thereafter, I briefly, but successfully investigated the epigenetics underlying personality traits in great tits. In 2013, I was awarded an NWO-Veni grant to further characterize *Nasonia dsx* and to study the evolutionary conservation of *dsx* in sexual and asexual parasitoids. I was the supervisor of a PhD student on the evolution of parasitoid sex determining mechanisms and I continued my work on the molecular basis of sex determination in *Nasonia*. Since 2015, I am a co-supervisor of a PhD student (EU-ITN BINGO) who works on the identification of genetic markers for improving biological control. From 2017 onwards, I am the daily supervisor of a PhD student who investigates the genetic basis of sex determination in an asexual parasitoid. The described proposal excellently builds up on these projects and will reinforce my research program considerably.

4g. International activities

Research visits

- Institute of Entomology, University of South Bohemia, Czech Republic (2015)
- Research institute of Insect Biology, University of Tours, France (2013)
- Institute of Developmental Biology, University of Cologne, Germany (2011)
- Institute of Entomology, University of South Bohemia, Czech Republic (2011)
- Institute of Development Genetics, New York University, USA (2009)
- Institute of Developmental Biology, University of Cologne, Germany (2009)
- Department of Biology, University of Rochester, USA (2007)

Presentations at international meetings/institutes

Summary: 8x invited speaker; 12x selected speaker; 3x poster

- Selected speaker. International *Nasonia* conference, Groningen, The Netherlands (2017). *Doublesex* regulates sex specific pigmentation in the parasitic wasp, *Nasonia vitripennis*.
- Selected speaker. Nöthiger meeting, Schiermonnikoog, The Netherlands (2017). *Doublesex* regulates sex specific pigmentation in the parasitic wasp, *Nasonia vitripennis.*
- **Invited speaker.** International Congress of Entomology, Orlando, USA (2016). The evolution of *doublesex* in two parasitic wasp genera: *Nasonia* and *Muscidifurax*.
- **Invited speaker.** Netherlands-Japan Seminar on Parasitoid Biology, Wageningen, The Netherlands (2014). Up and down the sex determining cascade of two parasitic wasps: *Nasonia* and *Muscidifurax*.
- Poster presentation. Conference of the Society for Molecular Biology and Evolution, San Juan, Puerto Rico (2014). DNA methylation of Dopamine Receptor D4 is associated with personality traits in great tits (*Parus major*).
- **Invited speaker.** Department seminar. Institut de Recherche sur la Biologie de l'Insecte, Tours, France (2013). A new upstream gene in the *Nasonia* sex determination cascade is maternally silenced and regulates *transformer* expression.
- Selected speaker. International *Nasonia* conference, Wageningen, The Netherlands (2013). Unfinished business in *Nasonia* sex determination.
- Selected speaker. Nöthiger meeting, Gif sur Yvette, France (2013). Unfinished business in *Nasonia* sex determination.
- **Invited speaker.** Job application for assistant professor 'Molecular Ecology', Freiburg, Germany (2013). Sex determination in Hymenoptera: the road after *doublesex*.
- Selected speaker. Nöthiger meeting, Amsterdam, The Netherlands (2012). Maternal imprinting regulates sex determination in the haplodiploid wasp, *Nasonia vitripennis*.
- Poster presentation. Conference of the European Society for Evolutionary Developmental Biology, Lisbon, Portugal (2012). Epigenetic control of *tra* expression regulates sex determination in the wasp *Nasonia*".
- Selected speaker. International *Nasonia* conference, Cologne, Germany (2012). Regulation of *tra* expression in *Nasonia vitripennis*.
- Selected speaker. International *Nasonia* conference, Nashville, USA (2011) *DNA methyltransferases* are crucial for embryonic development in *Nasonia*.
- **Invited speaker.** Institute seminar, University of South Bohemia, Czech Republic (2011). The genetic basis of sex determination in the haplodiploid wasp *Nasonia vitripennis*.
- Poster presentation. Sixth International Symposium on Molecular Insect Science, Amsterdam, The Netherlands (2011). DNA methylation plays a crucial role during

early development in the haplodiploid wasp *Nasonia*.
- Selected speaker. Annual Meeting of the Society for Molecular Biology and Evolution, Lyon, France (2010). Imprinting regulates sex determination in the haplodiploid wasp *Nasonia*.
- **Invited speaker.** Department seminar, University of New York, USA (2009). Sex determination in *Nasonia*: primary signal?
- Selected speaker. International *Nasonia* conference, Rochester, USA (2009). Sex determination in *Nasonia*: the facts
- **Invited speaker.** Department seminar, University of Cologne, Germany (2009). Sex determination in *Nasonia*: the facts.
- Selected speaker. Nöthiger meeting, Poros, Greece (2008). Sex determination in *Nasonia*.
- Selected speaker. International *Nasonia* conference, Edinburgh, United Kingdom (2008). Sex determination in *Nasonia.*
- **Invited speaker.** Department seminar, University of Rochester, USA (2007). Sex determination genes in *Nasonia*.
- Selected speaker. International *Nasonia* conference, Phoenix, USA (2007). The genetic basis of sex determination in *Nasonia*.

International collaborations

- Prof. J.H. Werren, Department of Biology, University of Rochester, USA
- Prof. C. Trent, Biology Department, Western Washington University, USA
- Prof. F. Vavre, Laboratoire de Biométrie et Biologie Evolutive, Université Lyon 1, France
- Prof. J.R. Gadau, Institute for Evolution & Biodiversity, University of Münster, Germany
- Dr. J.A. Lynch, Institute for Developmental Biology, University of Chicago, USA
- Dr. D. Bopp, Institute of Molecular Life Sciences, University of Zurich, Switzerland
- Dr. D. Doležel, Institute of Entomology, University of South Bohemia, Czech Republic

Conference organization

- Nöthiger meeting (International Sex Determination meeting), Schiermonnikoog, The Netherlands (2017).

Editor

- Invited guest editor for a special issue on "Sex determination and differentiation in insects" for Sexual Development, Vol. 8, No. 1-3 (2014). With Dr. Louis van de Zande

- Invited guest editor for a special issue on "The epigenetics of emerging and nonmodel organisms" for Genetics Research International, (2012). With Dr. Vett K. Lloyd, Dr. Jennifer A. Brisson, Dr. Kathleen A. Fitzpatrick and Dr. Lori A. McEachern

Appointment as a member of an expert panel

- Poster judge on the International *Nasonia* conference, Groningen (2017)
- Invited external expect member of PhD committee for Ellen Danneels, Ghent University, Belgium (2014)
- Referee for the evaluation of research proposals for Agence Nationale de la Recherche, France (2014)

- Poster judge on the Conference of the Society for Molecular Biology and Evolution, Puerto Rico (2014)

4h. Other academic activities

Presentations at national meetings/ institutes/ schools

- **Invited talk.** Yearly Laboratory of Entomology Meeting (YELREM), Wageningen University, The Netherlands. CRISPR/Cas9 gene editing (2017).
- **Invited lecture.** BSc course Philosophy of Science and Ethics, Wageningen University, The Netherlands. Gene drives in mosquito control (2017).
- Selected speaker. Netherlands Entomology meeting, Ede, The Netherlands. *Doublesex* regulates sex specific pigmentation in the parasitic wasp, *Nasonia vitripennis* (2016).
- **Invited talk.** Department seminar, Laboratory of Entomology, Wageningen University, The Netherlands. Evolution of sex determination in Hymenoptera: Study of two parasitic wasps, *Nasonia* and *Muscidifurax* (2016).
- **Invited speaker.** Dutch Society of Human Genetics Autumn Symposium, Papenburg, The Netherlands. Sex determination in Hymenoptera: the road after *doublesex* (2015).
- Selected speaker. Netherlands Entomology meeting, Ede, The Netherlands. *Doublesex* regulates sex specific dimorphism (2014).
- Selected speaker. Ecogenomics Day, The Platform Ecological and Evolutionary Genomics, The Netherlands. DNA methylation of Dopamine Receptor D4 is associated with personality traits in great tits (*Parus major*) (2014).
- Poster presentation. Netherlands Entomology meeting, Ede, The Netherlands. Male and female development: How does one gene do that? (2013).
- **Invited talk.** Institute seminar, Netherlands Institute of Ecology (2013).
- Poster presentation, Netherlands Institute of Ecology, Open dag. Epigenetica van koolmezen persoonlijkheid (2013).
- **Invited talk.** Job application for postdoctoral position, Netherlands Institute of Ecology. Maternal imprinting regulates sex determination in the parasitoid wasp, *Nasonia vitripennis* (2013).
- Selected speaker. Netherlands Entomology meeting, Ede, The Netherlands. Sex determination in *Nasonia*: males are womanizers (2012).
- **Invited lecture.** Evolutionary Genetics topmasters Biology, University of Groningen, The Netherlands. The genetic basis of sex determination in the haplodiploid wasp *Nasonia vitripennis*: Genomic imprinting is the key! (2011)
- Selected speaker. Netherlands Entomology meeting, Ede, The Netherlands. Imprinting regulates sex determination in the haplodiploid wasp *Nasonia vitripennis* (2010).
- **Invited lecture.** Medical en Farmaceutical Drug Innovation (UMCG, RuG) topmasters, University of Groningen, The Netherlands. The genetic basis of sex determination in the haplodiploid wasp *Nasonia vitripennis*: Genomic imprinting is the key! (2010)
- Selected speaker. Netherlands Entomology meeting, Ede, The Netherlands. Sex determination in *Nasonia* (2009).
- **Invited talk.** Center for Ecology and Evolution Studies, University of Groningen, The Netherlands. Sex determination in *Nasonia* (2009).
- Selected speaker. Netherlands Entomology meeting, Ede, The Netherlands. Sex determination in the haplodiploid jewel wasp *Nasonia* (2007).
- Selected speaker. Netherlands Entomology meeting, Ede, The Netherlands. Sex determination in the haplodiploid wasp *Nasonia vitripennis* (2006).

Popularizing science

I participate in the Wageningen University initiative "Wetenschapper in de Klas" (Science in the classroom) that aims to bring scientists to primary schools to teach children about science and empirical research. I take enormous pleasure in showing parasitoid wasps to children, to teach them about insects they normally do not see because of size. I explain how natural and sexual selection shape the biodiversity we see around us and we play a game to simulate empirical research that demonstrates how natural selection works.

Reviewer (since 2009)

Reviewing for: Scientific Reports; Heredity; Plos Genetics; Proceedings of the Royal Society B; Development, Genes and Evolution; Physiological Entomology; Plos One; Insect Molecular Biology; Animal Reproduction Science; FEBS Letters, Nature Ecology and Evolution

Science communication in the media

- Interview with national newspaper Volkskrant for item on "Vrouwtjesinsect ontdekt met penis en mannetjesinsect met vagina" (Discovery of female insect with penis and male insect with vagina) (2014).
- National newspaper NRC handelsblad – Wetenschapsbijlage "Sluipwesp laat ei onbevrucht en schakelt een gen uit om zoon te krijgen" (Parasitoid wasp doesn´t fertilize her eggs and shuts down a gene to obtain sons) (2011)
- University Newspaper (UK) "Moeders wil is wet" (Because mother says so) (2010).
- University Newspaper (UK) "1x sluipwesp = 2x Science" (1x parasitoid wasp = 2x Science) (2010).

Memberships of (inter)national networks, consortia or societies

- European Society for Evolutionary Biology (ESEB)
- The Netherlands Entomological Society (NEV)
- Insect Genetic Technologies Research Coordination Network (IGTRCN)
- Drosophila Parasitoids Consortium (Droparcon)
- *Nasonia* Genome Consortium

Teaching

Year	Course and level	ECTs	Students	Tasks	Institute
2016-present	MSc Fundamental and Applied Aspects of the Biology of Insects	6	±25	Supervision and grading of essays	WUR
2016-present	MSc Molecular Aspects of Bio-interactions	6	±100	Lecturing, coordinating and supervising student projects, grading reports and exam	WUR

2016-present	MSc Molecular and Evolutionary Ecology	6	±60	Co-coordinating course setup, supervising student projects and grading	WUR
2015-present	MSc thesis Biology	36	1	Supervision and grading	WUR
2015-present	BSc thesis/internship	24-36	4	Supervision and grading	WUR
2014	BSc Genetic Analysis, Tools and Concepts	6	100	Supervising student practical	WUR
2012-2013	BSc Evolutionary and Ecological Genetics	12	30	Supervising student computer practical	RuG
2012	MSc Molecular Methods in Ecology and Evolution	12	20	Supervising student projects	RuG
2006-2011	MSc thesis Biology	24-36	3	Supervision and grading	RuG
2006-2012	BSc Evolutionary and Ecological Genetics	12	20	Supervising student projects	RuG

***** WUR: Wageningen University; RuG: University of Groningen

4i. Scholarships, grants and prizes

Please list the research scholarships/grants for which you have successfully applied and/or prizes that you have won and indicate the amount of money involved.
**In case of a consortium grant please specify the amount awarded to your own group or lab.*

Scholarship/Grant/ Prize Formal applicant	**Amount in euros (k€)**	*****	**Year of award**
Poster award	0		2014
NWO-VENI	250		2013
Cum laude PhD thesis (top 5%)	0		2011
SMBE travel award	1		2010
UEF travel grant	1		2009
Subtotal	252		

Scholarship/Grant/Prize **Formal co-applicant**			
Subtotal	*252*		
Total	***252***		

Project grants

- 2013, NWO/ALW: € 250.000, highly competitive VENI talent grant for 3 years project on evolution of *doublesex* in parasitoids (Principal researcher E.C. Verhulst).

Travel grants

- Uyttenboogaart foundation grant for research visit to Prof. Desplan Lab at New York University to learn microinjections (€ 1000 in 2009).

Prizes

- *Cum laude* qualification for PhD thesis (2011), a very special qualification only provided to the top 5% of PhD theses.
- Poster award for poster entitled: "Epigenetics of great tit personality" at the Annual Meeting of the Dutch Society for Behavioural Biology (2014)
- Student Travel Award for participation on the Annual Meeting of the Society for Molecular Biology and Evolution (€1000 in 2010)

Output

5a. Output indicators

Please identify the most important output indicators in your field.

Output indicators for my field (also used by my University in evaluation of Tenure Track):

Publications:

- First authorship indicates primary author of the publication (impact factor >2.75 is considered a Quality 1 publication (top 25% of the field).
- Last author indicates senior author or PI on a project
- Individual publications are rare and often restricted to Opinion or Review papers, data publications are typically group or consortia publications
- Publications with international orientation are valued over national orientation
- Peer reviewed book chapters are valued as good peer reviewed journal publications, credits in Tenure Track equal Quality 2 publications (impact factor 1-3)

Other:

- Citation of papers published in peer-reviewed journals.
- PhD students successfully finishing their PhD research.
- Publication of (invited) reviews in prestigious journals
- Invited presentations at international conferences

5b. Top publications *(Max. five)*

Please motivate your selection.

Since my PhD, my research has focused on sex determination and differentiation in parasitoids and particularly *Nasonia*. I have elucidated the core components and their regulation of the *N. vitripennis* sex determination cascade[1], and supervised the functional identification of an additional sex determination gene[5]. I was involved in the genome assembly and annotation of *Nasonia*[2]; and I researched the role of *dsx* in regulating sex- and species-specific traits across insects[4]. In addition, I worked briefly on great tit epigenetics and behaviour[3]. With these five chosen titles I aim to show my independence and unique capacity to be the Principle Investigator for this proposal.

1. **Verhulst, E.C.**, Beukeboom, L.W., and van de Zande, L. (2010). Maternal control of haplodiploid sex determination in the wasp Nasonia. ***Science*** 328, 620–623. **IF = 37.2**
2. Werren J.H., Richards S., Desjardins C.A., … **Verhulst E.C.**, … (2010). Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. ***Science*** 327(5963): 343-348. **IF = 37.2**
3. **Verhulst, E.C.**, Mateman, C.A., Zwier, M.V., Caro, S.P., Verhoeven, K.J.F., and van Oers, K. (2015). Evidence from Pyrosequencing Indicates that Natural Variation in Animal Personality is Associated with DRD4 DNA Methylation. ***Molecular Ecology*** 28, 1801-1811. **IF = 6.1**
4. **Verhulst, E.C.**, and van de Zande, L. (2015). Double nexus--*Doublesex* is the connecting element in sex determination. ***Briefings in Functional Genomics*** 14, 396–406. *Invited review* **IF = 4.1**

5. Geuverink, E., Rensink, A.H., Rondeel, I., Beukeboom, L.W., van de Zande, L., and **Verhulst, E.C.** (2017). Maternal provision of *transformer-2* is required for female development and embryo viability in the wasp *Nasonia vitripennis*. ***Insect Biochemistry and Molecular Biology*** 90, 23-33. **IF = 3.8**

5c. Output

For publications: use reverse chronological order (i.e., newest first). Do not include any publications that have not yet been accepted for publication.

*Please number your items consecutively and mark key publications directly relevant to the proposed research with an S (the **S stands for significant**). Per category, indicate your total number of output items.*

Journal	Number	Impact factor (WoS 2016)
Science	2	37.2
Plos Genetics	1	6.1
Molecular Ecology	1	6.1
Current Opinion in Genetics & Development	1	5.8
Briefings in Functional Genomics	1	4.1
Insect Biochemistry and Molecular Biology	1	3.8
Insect Molecular Biology	3	2.8
Plos One	1	2.8
Sexual Development	2	2.0
	Total: 11	**Average: 8.9**

Refereed articles

1. Geuverink, E., **Verhulst, E.C.**, Van Leussen, M., Van de Zande, L., and Beukeboom, L.W. (2017). Maternal provision of non-sex-specific *transformer* messenger RNA in sex determination of the wasp *Asobara tabida*. ***Insect Molecular Biology*** *in press*. **IF = 2.8**; Cited 0

2. Geuverink, E., Rensink, A.H., Rondeel, I., Beukeboom, L.W., van de Zande, L., and **Verhulst, E.C.** (2017). Maternal provision of *transformer-2* is required for female development and embryo viability in the wasp *Nasonia vitripennis*. ***Insect Biochemistry and Molecular Biology*** 90, 23-33. **IF = 3.8**; Cited 0; **S**

3. **Verhulst, E.C.**, and van de Zande, L. (2015). Double nexus--*Doublesex* is the connecting element in sex determination. ***Briefings in Functional Genomics*** 14, 396–406. **IF = 4.1**; Cited 11; **S**

4. **Verhulst, E.C.**, Mateman, C.A., Zwier, M.V., Caro, S.P., Verhoeven, K.J.F., and van Oers, K. (2015). Evidence from Pyrosequencing Indicates that Natural Variation in Animal Personality is Associated with DRD4 DNA Methylation. ***Molecular Ecology*** 28, 1801-1811. **IF = 6.1**; Cited 15

5. Van de Zande, L., and **Verhulst, E.C.** (2014). Genomic imprinting and maternal effect genes in haplodiploid sex determination. ***Sexual Development*** 8, 74–82. **IF = 2.0**; Cited 12; **S**

6. **Verhulst, E.C.***, Lynch, J.A.*, Bopp, D., Beukeboom, L.W., and van de Zande, L. (2013). A new component of the *Nasonia* sex determining cascade Is maternally silenced and regulates *transformer* expression. ***PLoS ONE*** 8, e63618. **IF = 2.8**; Cited 14

7. Zwier, M.V., **Verhulst, E.C.**, Zwahlen, R.D., Beukeboom, L.W., and van de Zande, L. (2012). DNA methylation plays a crucial role during early *Nasonia* development. ***Insect Molecular Biology*** 21, 129–138. **IF = 2.8**; Cited 32

8. Loehlin, D.W., Oliveira, D.C.S.G., Edwards, R., Giebel, J.D., Clark, M.E., Cattani, M.V., van de Zande, L., **Verhulst, E.C.**, Beukeboom, L.W., Muñoz-Torres, M., et al. (2010). Non-coding changes cause sex-specific wing size differences between closely related species of *Nasonia*. ***PLoS Genetics*** 6, e1000821–e1000821. **IF = 6.1**; Cited 38; **S**

9. Werren J.H., Richards S., Desjardins C.A., Niehuis O., Gadau J., Colbourne J.K. Beukeboom L.W., Desplan C., Elsik C.G., Grimmelikhuijzen C.J., Kitts P, Lynch J.A., Murphy T., Oliveira D.C., Smith C.D., van de Zande L., Worley K.C., Zdobnov E.M., Aerts M., Albert S., Anaya V.H., Anzola J.M., Barchuk A.R., Behura S.K., Bera A.N., Berenbaum M.R., Bertossa R.C., Bitondi M.M., Bordenstein S.R., Bork P., Bornberg-Bauer E., Brunain M., Cazzamali G., Chaboub L., Chacko J., Chavez D., Childers C.P., Choi J.H, Clark M.E, Claudianos C., Clinton R.A., Cree A.G., Cristino A.S., Dang P.M., Darby A.C., de Graaf D.C., Devreese B., Dinh H.H., Edwards R., Elango N., Elhaik E., Ermolaeva O., Evans J.D., Foret S., Fowler G.R., Gerlach D., Gibson J.D., Gilbert D.G., Graur D., Gründer S., Hagen D.E., Han Y., Hauser F., Hultmark D., Hunter H.C. 4th, Hurst G.D., Jhangian S.N., Jiang H., Johnson R.M., Jones AK., Junier T., Kadowaki T., Kamping A., Kapustin Y., Kechavarzi B., Kim J., Kim J., Kiryutin B., Koevoets T., Kovar C.L., Kriventseva E.V., Kucharski R., Lee H., Lee S.L., Lees K., Lewis L.R., Loehlin D.W., Logsdon J.M. Jr., Lopez J.A., Lozado R.J., Maglott D., Maleszka R., Mayampurath A., Mazur D.J., McClure M.A., Moore A.D., Morgan M.B., Muller J., Munoz-Torres M.C., Muzny D.M., Nazareth L.V., Neupert S., Nguyen N.B., Nunes F.M., Oakeshott J.G., Okwuonu G.O., Pannebakker B.A., Pejaver V.R., Peng Z., Pratt S.C., Predel R., Pu L.L., Ranson H., Raychoudhury R., Rechtsteiner A., Reese J.T., Reid J.G., Riddle M., Robertson H.M., Romero-Severson J., Rosenberg M., Sackton T.B., Sattelle D.B., Schlüns H., Schmitt T., Schneider M., Schüler A., Schurko A.M., Shuker D.M., Simões Z.L., Sinha S., Smith Z., Solovyev V., Souvorov A., Springauf A., Stafflinger E., Stage D.E., Stanke M., Tanaka Y., Telschow A., Trent C., Vattathil S., **Verhulst E.C.**, Viljakainen L., Wanner K.W., Waterhouse R.M., Whitfield J.B., Wilkes T.E., Williamson M., Willis J.H., Wolschin F., Wyder S., Yamada T., Yi S.V., Zecher C.N., Zhang L., Gibbs R.A. (2010). Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. ***Science*** 327(5963): 343-348. **IF = 37.2**; Cited 541; **S**

10. **Verhulst, E.C.**, Beukeboom, L.W., and Van de Zande, L. (2010). Maternal control of haplodiploid sex determination in the wasp *Nasonia*. ***Science*** 328, 620–623. **IF = 37.2**; Cited 121; **S**

11. **Verhulst, E.C.**, Van de Zande, L., and Beukeboom, L.W. (2010). Insect sex

determination: it all evolves around *transformer*. ***Current Opinion in Genetics & Development*** 20, 376–383. **IF = 5.8**; Cited 103

12. Oliveira, D.C.S.G., Werren, J.H., **Verhulst, E.C.**, Giebel, J.D., Kamping, A., Beukeboom, L.W., and van de Zande, L. (2009). Identification and characterization of the *doublesex* gene of *Nasonia*. ***Insect Molecular Biology*** 18, 315–324. **IF = 2.8**; Cited 62; **S**

(* equal contribution)

Non-refereed articles

1. **Verhulst, E.C.**, and van de Zande, L. (2014). Insect Sex Determination: A Cascade of Mechanisms. ***Sexual Development*** 8, 5–6. *Preface*

2. Lloyd, V.K., Brisson, J.A., Fitzpatrick, K.A., McEachern, L.A., and **Verhulst, E.C.** (2012). The Epigenetics of Emerging and Nonmodel Organisms. ***Genetics Research International*** 2012, 1–2. *Preface*

Summary		
Total publications	14	
Publications in international refereed journals	12	
Non-refereed articles	2	
	Google Scholar	**Web of Science**
My H-index	10	8
Total cites	956	700

5d. Median impact factors for your field

This section is compulsory only if you have mentioned journal impact factors in section 5c, above.

Research field	Median Impact Factor
Evolutionary Biology	2.5
Genetics & Heredity	2.5
Biochemistry & Molecular Biology	2.8
Entomology	1.0
Ecology	1.9
Developmental Biology	2.3

Statements by the applicant

Use of extension clause:

☐ Yes
☑ No

(if 'yes', give reasons,calculation and the date of the NWO email granting you the extension)

Ethical aspects

	Not applicable	Not yet applied for	Applied for	Received
Approval from a recognised (medical) ethics review committee	**X**			
Approval from an animal experiments committee	**X**			
Permission for research with the population screening Act	**X**			

If your grant application is successful, all applicable ethical approval documents will need to be sent to NWO before the start of your Vidi can start.

Declarations

By submitting this form, I endorse the code of conduct for laboratory animals and the code of conduct for biosecurity/possibility for dual use of the expected results and will act accordingly, if applicable.

☑ **I have completed this form truthfully**

By submitting this document I declare that I satisfy the nationally and internationally accepted standards for scientific conduct as stated in the Netherlands Code of Conduct for Scientific Practice (Association of Universities in the Netherlands)

☐ I have submitted non-referees.*

☑ If applicable: I have included one or more authorised letters from the prospected host institution and/or a third party, guaranteeing to meet part of the costs of this research project.

Name:

Place: Wageningen

Date: 4 October 2017

* You may indicate non-referees (a maximum of three names) **in ISAAC** (or, for ZonMw, by email directly to vidi@ZonMw.nl). The non-referees will NOT be asked to assess your application. Please do **not** incorporate the names of your non-referees in this application form.

Please submit this application form to NWO in PDF format (and please do not use any security locks or bookmarks in the PDF file), using ISAAC, which can be accessed at isaac.nwo.nl, or, if you are applying to ZonMw, using ProjectNet, which can be accessed through http://www.zonmw.nl.

For any technical questions regarding submission, please contact the ISAAC helpdesk (Isaac.helpdesk@nwo.nl) or the ProjectNet helpdesk (projectnet@zonmw.nl), respectively.