# Phenotypic Screen and Transcriptomics Approach Complement Each Other in Functional Genomics of Defensive Stink Gland Physiology

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## Abstract

# Background

Functional genomics uses unbiased systematic genome-wide gene disruption or analyzes natural variations such as gene expression profiles of different tissues from multicellular organisms to link gene functions to particular phenotypes. Functional genomics approaches are of particular importance to identify large sets of genes that are specifically important for a particular biological process beyond known candidate genes, or when the process has not been studied with genetic methods before.

# Results

Here, we present a large set of genes whose disruption interferes with the function of the odoriferous defensive stink glands of the red flour beetle *Tribolium castaneum*. This gene set is the result of a large-scale systematic phenotypic screen using a reverse genetics strategy based on RNA interference applied in a genome-wide forward genetics manner. In this first-pass screen, 130 genes were identified, of which 69 genes could be confirmed to cause knock-down gland phenotypes, which vary from necrotic tissue and irregular reservoir size to irregular color or separation of the secreted gland compounds. The knock-down of 13 genes caused specifically a strong reduction of para-benzoquinones, suggesting a specific function in the synthesis of these toxic compounds. Only 14 of the 69 confirmed gland genes are differentially overexpressed in stink gland tissue and thus could have been detected in a transcriptome-based analysis. Moreover, of the 29 previously transcriptomics-identified genes causing a gland phenotype, only one gene was recognized by this phenotypic screen despite the fact that 13 of them were covered by the screen.

# Conclusion

Our results indicate the importance of combining diverse and independent methodologies to identify genes necessary for the function of a certain biological tissue, as the different approaches do not deliver redundant results but rather complement each other. The presented phenotypic screen together with a transcriptomics approach are now providing a set of close to hundred genes important for odoriferous defensive stink gland physiology in beetles.

## Background

Functional genomics uses unbiased genome-wide approaches to identify the biological function of genes based on high-throughput or large-scale experimental methodologies [1]. To link gene functions to particular phenotypes, functional genomics uses systematic gene disruption or analyzes natural variations such as gene expression profiles of different tissues from multicellular organisms [2]. Therefore, functional genomics approaches make it possible, to identify sets of genes that are

specifically important for the development and physiology of a certain tissue. Such approaches are of particular importance to complete the knowledge beyond candidate genes, or when a certain tissue has not yet been studied with genetic methodologies before.

Beetles and ants are the most prolific producers of defensive substances [3], which are usually multifunctional and operate as repellents, surfactants, antimicrobics, or toxicants against a large array of potential target organisms [4]. Many Coleoptera biosynthesize and store their defensive compounds in complex glands and release them by controlled opening of the gland reservoirs [5]. In *Tribolium* beetles (Coleoptera: Tenebrionide), odoriferous defensive stink glands [6] are present in pairs in the caudal abdomen (posterior, abdominal, or pygidial glands) and in the prothorax (anterior, thoracic, or prothoracic glands). The glands are composed of two types of secretory units with particular vesicular organelles, tubules, duct, a reservoir and respective muscles [6–8]. In the glands of Tenebrionid beetles, highly reactive, unstable, and toxic para-benzoquinone compounds are produced in large amounts [5, 6, 8–12] to condition their microenvironment [13]. Particular interest on secretions of *Tribolium* beetles came about, since their conditioning of flour turns it pink [14] and renders it unusable and hazardous to human health [15, 16]. Major secretion components besides the toxic para-benzoquinones are 1-alkenes also called terminal olefins [17–21]. These represent extremely versatile chemical intermediates and thus serve as important products with direct application in the production of biofuels or other industrial chemicals such as plasticizers, emulsifiers, or biodegradable surfactants [22].

For *Tribolium* stink glands, more than fifty years ago, histological and biochemical techniques revealed some basic insights into the process of toxic para-benzoquinone production [8] and about 30 years ago into terminal olefin biosynthesis [20]. However, no more details on how the different enzymatic activities are localized to the different compartments have so far been obtained. Howard already pointed out almost twenty years ago [23], that molecular genetics has so far dealt little with this topic despite its great potential to help to better understand semiochemical and defensive compound secretion. Even today not much is known about the genes that are required for regulating and executing the production of defensive secretions or for self-protection mechanisms against auto-intoxication by the defensive compounds. This is probably because the main genetic insect model, *Drosophila melanogaster*, does not have such glands. Recently, however, the red flour beetle, *T. castaneum*, has emerged as a genetic model organism to study development, physiology and coleopteran pest biology with an array of tools available for functional genetic work [24]. *T. castaneum* carries defensive glands and at least some genetic data on gland physiology come from this species with three mutant strains carrying visibly gland phenotypes: *melanotic stink glands* (*msg*; [25]), *tar* [26], and *box* ($A^{box}$; [27]). The mutations affect para-benzoquinone secretion and result in modification of the substances contained in the reservoirs. Moreover, a first functional genomics approach based on transcriptome analysis identified 77 genes in this species that were 64x higher expressed in the gland tissue compared to an abdominal control tissue. Of these, 71 genes were functionally analysed by RNA interference-mediated (RNAi) gene knock-down in respect to their necessity for gland morphology and function.

The functional genetic tools in *T. castaneum* include forward genetics based on insertional mutagenesis [28], transgene-based miss-expression systems [29, 30], a fully annotated genome sequence [31–33], as well as systemic RNAi [34, 35]. The efficient use of RNAi in this beetle has allowed the development of this reverse genetics approach into a forward genetics application to perform an unbiased genome-wide large-scale phenotypic screen (iBeetle screen) to identify gene functions in embryonic and postembryonic development as well as cell biology and physiology [36, 37]. In this screen, the knock-down situations were besides other phenotypes systematically checked for phenotypes in the stink glands and methodically documented in the iBeete-Base [38, 39]. With the iBeetle screen, we were for the first time able to identify a large set of genes required for stink gland function directly based on phenotypes. We used the iBeetle-Base documentation of this first first-pass screen to re-screen all the genes identified for potentially affecting gland formation and function and could validate out of 130 genes that for 69 genes, the knock-down indeed causes gland phenotypes. Here, we present the genes of this unbiased phenotypic screen with their phenotypes and compare the results with the previously identified stink gland gene set using transcriptome analysis based on tissue-specific RNAseq analysis [40].

# Results And Discussion

### iBeetle: Large-scale genome-wide phenotypic screen

iBeetle stands for a large-scale systemic RNAi-based screen using the red flour beetle, *T. castaneum*, as a screening platform. Double-stranded RNA injections were performed in larval or pupal stages and the phenotypic effects scored at multiple levels such as cell biology, physiology, or embryonic and postembryonic development [36, 37]. In the first and second phase of iBeetle, together about 8500 gene models were screened for their function by systematic gene knock-down, which corresponds to slightly more than 50% of the currently annotated genes in this emerged model organism [33]. The obtained phenotypes were documented in iBeetle-Base [38, 39]. The phenotypic analysis included the detection of visible morphological changes affecting the odoriferous stink glands of this beetle, however, without detailed investigation by systematic dissection. Thus, iBeetle serves as a crude first-pass screen to identify potentially interesting genes for a particular biological process. These genes need then to be verified by a detailed re-screening process with a particular focus on the tissue of interest. For 130 genes, corresponding to about 1,5% of the analyzed gene models, phenotypic changes in gland morphology were noted in iBeetle-Base. These were subject to a re-screen procedure based again on RNAi, and for 69 of them (53%), a knock-down gland phenotype could be detected (Additional file 1: Supplementary Table 1). In 60 cases, the original iBeetle-identified phenotype could be confirmed, whereas in nine cases (all from the second iBeetle phase) a distinctive gland phenotype was detected (Table 1). While the iBeetle screen was using a transgenic enhancer trap strain (Pig-19) [41], the re-screen was performed in the wild type San Bernadino strain. The difference in the observed phenotypes might thus be caused by strain-specific differences, which have previously been observed in *T. castaneum* RNAi-induced phenotypes [42].

### Morphological knock-down phenotypes of *Tribolium castaneum* stink glands

During the first two phases of the iBeetle screen, nine different screeners checked systematically for phenotypes in respect to embryonic and postembryonic development as well as cell biology and physiology. Therefore, the phenotype descriptions noted in iBeetle-Base are quite variable and not completely standardized (Additional file 1: Supplementary Table 1). For the re-screen of all 130 genes with a noted gland phenotype, the visible morphological phenotypes were categorized into seven groups (Figure 1): glands empty and/or necrotic (Figure 1B); reservoir size irregular (increased or decreased, Figure 1E) or containing less secretion (Figure 1H); color of the secretion either darker (Figure 1C), melanized (Figure 1D), colorless (Figure 1F), or showed an irregular separation of the gland compounds (Figure 1G). An additional gland phenotype, that was not observed in the iBeetle screen or re-screen, is turbid secretion (Figure 1I), which was detected in the knock-down of one of the transcriptomics-identified genes (Tc_003768) and originally described as “condensed” (GT12, [40]. The knock-down of this particular gene causes a rare alkene-less phenotype not affecting the benzoquinone production [40], which has so far not been observed for any other gene knock-down. The comparison of the originally described gland phenotypes in the iBeetle screen with the categorized phenotypes of the re-screen is provided in Additional file 1: Supplementary Table 1. For the 69 genes with a re-screen confirmed gland phenotype, the gland morphology category is also indicated in Table 2.

For 61 genes, the iBeetle-Base originally noted phenotype could not be reproduced even with injections of higher concentrations of dsRNA. The majority of the annotated stink gland phenotypes for these genes are hard to identify (‘less secretion’, ‘irregular reservoir size’, and ‘secretion color darker’), as also in wild type beetles, stink gland secretions display natural variation in the degree of filling, color, and shape. Therefore, it is highly likely that these genes were assigned as false-positives in the first-pass iBeetle screen that needs re-screening for confirmation.

### Changes in stink gland volatile compounds of gland gene knock-downs

To examine whether the knockdown of the 69 re-screen-identified genes not only caused a visible morphological phenotype but also a change in gland contents, we applied gas chromatography-mass spectrometry to analyze the volatile compounds of the gland secretions independently for the thoracic and abdominal glands. In wild type beetles, the volatile secretion composition is similar in males and females [40], with the four main volatiles corresponding to two para-benzoquinones, 2-Methyl-1,4-benzoquinone (MBQ) and 2-Ethyl-1,4-benzoquinone (EBQ), and two alkenes, 1-Pentadecene (1-C15) and 1-Heptadecene (1-C17). In the different gene knock-downs, the abundances of the four main volatiles were altered to different degrees, reaching from higher than wild type levels, to no alteration, and down to undetectable levels of all or specific compounds (Table 2). The two para-benzoquinones or the two alkenes were usually affected together, whereas the production of the two compound groups seems to be independent. Of the 69 genes analyzed, we found in total that 51 showed strong volatile changes (≥ 50% reduction) in at least one type of compounds (para-benzoquinones or alkenes), in at least one gender or one type of gland. Very strong reductions of both benzoquinones and alkenes in thoracic and abdominal glands independent of the beetle’s sex were observed in the the knock-down of nine genes, which, however, gave rise to very different morphological gland phenotypes (colorless, irregular size,

empty/necrotic, darker, melanized). This indicates that the lack of volatile compounds can be the result of very different causes.

Interestingly, 13 gene knock-downs caused specifically a strong reduction of para-benzoquinones levels only, suggesting a specific function in the synthesis of these compounds. In contrast, none of the 69 gene knock-downs led to a specific reduction in alkene production only. In ten cases, reductions of volatile levels were observed to be gland-type specific (six thoracic, four abdominal), and for seven genes the secretion phenotype was found to be sex-specific (two in male, five in female). However, it should be noted that measurements have been done only once with a small number of individuals and gland- or sex-specific effects will have to be confirmed in future experiments. For three genes (iB_04797, iB_05441, iB_09043), whose knock-down caused the morphological phenotype 'colorless', also a lack of benzoquinones for both sexes and in both types of glands was observed, linking the yellowish color in wild type stink glands to the presence of para-benzoquinones in the secretions. Besides that, no direct correlation between the visible morphological phenotype and the secretion volatile compound phenotype could be recognized (Table 2).

## Encoded molecular functions of genes required for stink gland physiology

To identify the molecular function of the 69 functionally rescreen-confirmed gland genes, the nucleotide or amino acid conserved domains were identified using the respective National Center for Biotechnology Information (NCBI) online search tool [43] and homologs were screened for specifically in *D. melanogaster* and the entire NCBI nucleotide collection database. The results are provided are provided in Additional file 2: Supplementary Table 2. As expected for a secretory tissue, twenty genes encode enzymes, six transporters, and three channels (Figure 2). However, also two transcription factors were identified as well as thirty diverse other molecular functions, mainly receptors and enzyme regulators. For eight genes (11,6%), no molecular function could be assigned so far, which is probably due to the fact that a systematic genome-wide phenotypic analysis of an odoriferous stink gland tissue has not been performed before.

## Transcriptome-based expression levels of iBeetle-identified gland genes

To examine, whether the phenotypically detected genes during the iBeetle screen could have also been identified by a functional genomics approach based on transcriptomics analysis, we identified their expression levels in the published *Tribolium* stink gland transcriptome [40]. Out of the 130 originally identified genes, only 17 have a more than two times higher expression in the gland tissue (Additional file 1: Supplementary Table 1) and about a third have even a clearly reduced level of expression in the gland tissue compared to a control tissue (Additional file 3: Supplementary Table 3). Thus, the phenotypic screen can also identify genes that are not specifically active in the glands but also in other tissues, but are still necessary for the function of the glands. Of the 69 re-screen-confirmed gland genes, only 14 (20%; Table 1) and of 51 gland genes with a strong secretion phenotype, only nine genes (Table 2) show a two-fold or higher (FC $\geq$ 2) expression in the gland tissue. This indicates that about 80% of the genes with a

function in stink gland physiology have been missed using differential gene expression data to select candidate genes.

### Transcriptome-identified gland genes covered by the iBeetle screen

In the odoriferous defensive stink gland transcriptome data, Li et al. [40] identified 77 genes that are highly and specifically expressed in the stink glands (FC ≥ 64). 71 of them were analyzed in gene knock-downs for visible morphological and secretion volatile stink gland phenotypes. Out of these 71 genes, 36 were also analyzed during the first and second phase of the iBeetle screen (Figure 3). Only 29 of the 71 genes showed morphological and secretion volatile phenotypes in the stink glands (Additional file 4: Supplementary Table 4) [40]. Of these 29, 13 were covered in the first and second phase of the iBeetle screen. However, from these 13, only one gene (iB_09413), which caused a melanized phenotype, was confirmedly identified (conf.) also in the iBeetle screen. Two (iB_5763 and iB_5847) were not analyzed (n.a.), since they belonged to the 800 genes that were covered in the pupal screen of the first phase of iBeetle but not in the larval screen, which covered the stink gland analysis. Three genes covered in the first phase of iBeetle caused a lethal phenotype probably due to the larval injection, since the functional analysis of the transcriptome-identified genes was analyzed by pupal injections [40]. For seven genes, the gland phenotypes were not detected (n.d.) in the iBeetle screen despite that fact that they were analyzed. For six of these genes, the phenotypes are hard to detect (less secretion, colorless, turbid, empty/necrotic) and could have easily be missed by the diverse screeners of iBeetle (Additional file 4: Supplementary Table 4). However, one gene (iB_07205) causes a melanized phenotype that should have been detected in the iBeetle screen. In summary, the iBeetle screen identified only one out of eight gland genes that had been identified in the transcriptomics-based approach by Li et al. [40] and were functionally analyzed during the iBeetle screen (Figure 3).

## Conclusion

*T. castaneum* is a significant worldwide pest beetle of stored grains. It produces and releases defensive secretions acting as toxic, repellent, bacteriostatic, and fungistatic oils containing substituted para-benzoquinone compounds. To identify a large set of genes that are specifically important for such a particular tissue that had not been studied with genetic methods before, our present study indicates that one needs to combine several diverse functional genomic approaches. In a previous transcriptomics-based analysis [40], 77 genes were identified to be highly and specifically expressed in the gland tissue and for 29 of them it could be shown, that a knock-down causes a visible morphological gland phenotype. In our present study, we could identify 69 genes by a phenotypic screen to cause such altered glands. Only one gene was identified in both screens, indicating the different approaches do not deliver redundant results but rather complement each other. Both functional genomics approaches together are now providing a set of almost hundred genes (97) that have been shown to be necessary for odoriferous defensive stink gland physiology in beetles.

## Methods

## *Tribolium* rearing

*T. castaneum* (Herbst, 1797; Insecta, Coleoptera, Tenebrionidae) strains were reared on organic wheat flour supplemented with 5% yeast powder at 28°C and 40% relative humidity under constant light. The Beetles were collected from different breeding boxes varying in age (up to three month) and culture density.

## iBeetle first phase: screening and rescreening:

In the first phase of the iBeetle screen, which covered 5300 genes, potential gland phenotypes were analyzed in the 'larval screen' part, in which penultimate larvae (L6) of a particular cross [36] were injected with 1 µg/µl double-stranded RNA of the respective iBeetle fragment (https://ibeetle-base.uni-goettingen.de/) [38, 39]. The glands were then inspected at the adult stage 38 to 41 days after injection for aberrant gland phenotypes. However, the larval screen actually only covered about 4500 genes, resulting in 800 genes that were not screened for involvement in gland function. In iBeetle-Base [38], 57 genes were noted to probably have a function in gland physiology, for which gene fragments were identified that did not overlap with the original iBeetle screen fragment (Additional file 5: Supplementary Table 5). To confirm the knock-down-induced gland phenotype of these genes, double-stranded RNA of non-overlapping fragments were injected at a concentration of 2 to 3 µg/µl into pupae or larvae of the wild type San Bernadino strain. For 32 genes the iBeetle-identified phenotype could be confirmed (Additional file 1: Supplementary Table 1).

## iBeetle second phase: screening and rescreening

In the second phase of the iBeetle screen, which covered 3400 genes, double-stranded RNA injection of iBeetle fragments (https://ibeetle-base.uni-goettingen.de/) [38, 39] was performed at a concentration of 1 µg/µl into pupae of the PIG-19 strain [41]. Stink gland analysis was carried out at the adult stage 21 days after injection [37]. For the second phase, an additional 73 genes were noted in iBeetle.Base [39] to potentially have a knock-down-mediated altered phenotype. These 73 genes were re-screened by injecting the original iBeetle screen fragment again into pupae of the wild type San Bernadino strain at a concentration of 1 µg/µl. For 28 genes the iBeetle-identified gland phenotype could be confirmed, while for nine genes a gland phenotype could be detected that did not match the original described phenotype (Additional file 1: Supplementary Table 1). Based on the morphological (Figure 1; Additional file 1: Supplementary Table 1) and the volatile gland compound phenotype (strong reduction in benzoquinone synthesis; Table 2), for 19 genes – 18 confirmed plus one non-matching gland phenotype (iB_07902) – also non-overlapping fragments were identified (Additional file 6: Supplementary Table 6). After double-stranded RNA injection of these at a concentration of 2 µg/µl into wild type San Bernadino strain pupae, the re-screen phenotype could be confirmed for all 19 genes.

## Sequences, genome assemblies, and gene identifiers used

The first two phases of the iBeetle screen started on the knowledge of the draft genome assembly Tcas3.0 [31] and the official gene set (OGS) 2 [32]. The sequences used for the non-overlapping fragments for the re-screens are thus based on Tcas3 (Additional file 5: Supplementary Table 5 and Additional file 6: Supplementary Table 6). The gene function analysis (Additional file 1: Supplementary Table 1) and the gene ontology analysis (Additional file 2: Supplementary Table 2) are based on the current OGS 3 and genome assembly Tcas5.2 [33] with changes in Tc gene numbers between Tcas3 and Tcas5.2 indicated in Additional file 1: Supplementary Table 1. In all tables but one, the genes are sorted by the unique iBeetle number (https://ibeetle-base.uni-goettingen.de/) [38, 39] and cross-referencing to the Tc gene number is provided. Only in Additional file 4: Supplementary Table 4, the genes are listed based on the Tc numbers derived from Tcas3.0, which was the genome assembly used at the time of the transcriptome analysis [40], but annotation notes regarding changes into genome assembly Tcas5.2 are provided. The transcriptome analysis [40] is also the reason, why in Additional file 3: Supplementary Table 3, the iBeetle numbers are referring to the Tc numbers of assembly Tcas3.0.

## Double-stranded RNA synthesis and injection

In the re-screening of the first phase of iBeetle, double stranded RNAs were self-designed using the E-RNAi web service of the German cancer research center [44] to identify best fragments without off-targets. The fragments were cloned and the *in vitro* transcription was performed with the MEGAscript® T7 Kit from Ambion® (Life Technologies GmbH, Darmstadt, Germany, Cat#: AM1334) using the purified PCR product of the respective gene fragment with added T7-RNA Polymerase promoter sites as template DNA. In the re-screening of the second phase of iBeetle, original iB-fragments and non-overlapping fragments at a concentration of 3 µg/µl were ordered from Eupheria Biotech GmbH (Dresden, Germany). The synthesized double stranded RNAs were re-suspended and diluted in injection buffer (10x stock: 14 mM NaCl, 0.7 mM Na2HPO4 • 2H2O, 0.3 mM KH2PO4, 40 mM KCl) and stored at -20°C.

Double stranded RNA injections were usually carried out at mid-pupal stage of male and female beetles. Before injection, pupae were incubated for 5 min on ice and then lined up on an adhesive tape placed on a microscope glass slide. Borosilicate glass capillaries (length: 100 mm, outside-diameter: 1 mm, wall thickness: 0.21 mm, Heinemann Labortechnik GmbH, Duderstadt, Germany) were pulled with the Micropipette Puller Model P-2000 (Sutter Instruments, Novato, USA) to generate injection needles. For semiautomatic injections, a FemtoJet®express microinjector (Eppendorf, Hamburg, Germany) was used in combination with a micromanipulator (M1, Helmut Saur Laborbedarf, Reutlingen, Germany). The injection was carried out under a stereomicroscope.

## Phenotypic analysis of knock-down beetle stink glands

In the re-screen, the prothoracic and abdominal stink glands were dissected from wild type and knock-down beetles about three weeks after injection. The gland and reservoir morphology was analyzed under a dissection stereomicroscope (Leica MZ16FA) and exemplary pictures of the diverse phenotypes were taken with a Q-imaging camera (Leica Microsystems GmbH, Wetzlar, Germany). Pictures were processed with Adobe Photoshop CS2 (Figure 1).

The gland content volatiles from the prothoracic and abdominal gland tissues of knockdown and control (wild type) beetles were independently analyzed by semi-quantitative gas chromatography-mass spectrometry (GC-MS). In the beginning of re-screening in the 1st phase of the iBeetle screen, a mixture of the gland secretions of one female and one male beetle was analyzed, while later on and in the re-screening in the 2nd phase of iBeetle, male and female beetles were analyzed separately. The abdominal and prothoracic glands were collected from ten day old adult beetles and homogenized in 50 µl (one beetle) or 100 µl (three beetles) methanol (Merck Millipore KGaA, Darmstadt, Germany). The processed gland tissue samples were stored on -20°C before the GC-MS analysis, which was performed within 48 h. Per sample,1 µl was loaded by a split injector into a GC-MS system composed of a gas chromatograph (6890N Network GC System, Agilent Technologies, Santa Clara, USA) and a mass spectrometer (5973 Network Mass Selective Detector, Agilent Technologies, Santa Clara, USA) connected to a MultiPurposeSampler (MPS, Gerstel, Mülheim, Germany). The GC-MS data were analyzed by the MSD ChemStation D.02.00.275 software (Agilent Technologies, Santa Clara, USA). The volatile secretion compounds were identified with the NIST 2008 and Wiley 9th edition databases (National Institute for Standards and Technology, Gaitherburg, USA; Wiley, Hoboken, USA). The calculations of semi-quantitative analysis of volatile gland secretion substances and comparative chromatograms display were done in Microsoft Excel in both wild type and knock-down situations. For these analyses, first the mean values of abundances of wild type beetle gland chemicals (buffer injected) were determined and set as 100%. Subsequently the relative alterations in secretion substances in gene knock down beetle glands in relation to the wild type mean were calculated in percent, in which values >100% indicate an increase of the respective substance in the knockdown glands compared to the wild type and values <100% identify a corresponding reduction. Of the gland volatiles, the two most abundant para-benzoquinones (2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone) and the two most abundant alkenes (1-pentadecene and 1-heptadecene) were analyzed (Table 2).

### Homolog search and identification of conserved domains

The encoded proteins of the 69 identified genes were screened with BLASTp against the *Tribolium* genome assembly Tcas5.2 [45] and with tBLASTn against the *D. melanogaster* genome and the entire NCBI nucleotide collection database. Moreover, the nucleotide or amino acid conserved domains were identified using the respective NCBI online search tool [43]. In addition, the gene ontology annotation regarding molecular function [46] was determined (Figure 2). The respective results are provided in Additional file 2: Supplementary Table 2.

# Abbreviations

1-C15 1-pentadecene

1-C17 1-heptadecene

conf. confirmed

| | |
|---|---|
| dsRNA | double-stranded RNA |
| EBQ | 2-ethyl-1,4-benzoquinone |
| FC | Fold change |
| GC-MS | Gas chromatography-mass spectrometry |
| MBQ | 2-methyl-1,4-benzoquinone |
| n.a. | not analyzed |
| n.d. | non detected |
| NOFs | Non-overlapping fragments |
| OGS | Official gene set |
| RNAi | RNA interference |
| RNA-Seq | Next generation sequencing of mRNA |
| Tcas3.0 | Official assembly of genomic sequence of *Tribolium castaneum* version 3 0 |
| Tcas5.2 | Official assembly of genomic sequence of *Tribolium castaneum* version 5.2 |

# Declarations


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### Authors' contributions

E.A.W conceived the study; G.B., C.S-E., and M.K. designed the iBeetle screen procedure; D.G., C.S-E., N.S., U.M., T.R., M.W., S.A., M.T., and M.S.H. carried out the first-pass iBeetle screen to identify potential gland phenotypes; S.L. and B.A. performed the re-screen and functional analysis of the confirmed genes with help of B.W. regarding GC-MS equipment; J.L. analyzed the gland transcriptome; S.L., B.A, and E.A.W prepared the figures and wrote the manuscript; all authors read and approved of the final manuscript.


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South-East-West Mobility. The APC was funded by the Open Access Publication Funds of the Göttingen University. All funding bodies had no role in the design of the study, the collection, analysis, and interpretation of data, or in writing the manuscript.

### Availability of data and materials

The datasets of the iBeetle screen including the fragments used as double-strand RNAs for the gene knock-downs are available in iBeetle-Base (https://ibeetle-base.uni-goettingen.de/) [38; 39]. All other data analysed and generated during this study are cited, or included in this published article and its supplementary information files, respectively.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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# Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

# Figures

### Figure 1

Visible morphological stink gland phenotypes identified in specific gene knock-downs. Morphologies differing from wild type (A) were categorized into seven groups: glands empty and/or necrotic (B); color of secretion darker (C); color of secretion melanized (D); reservoir size irregular (E); colorless secretion (F); irregular separation of gland compounds (G); containing less secretion (H); or turbid secretion (I), a phenotype that was not observed in the iBeetle screen or re-screen, but was detected in the knock-down of one of the transcriptomics-identified genes (Tc_003768) and originally described as “condensed” [40]. The iBeetle numbers of the representative gene knock downs the pictures were taken of are indicated.

### Figure 2

Molecular functions of confirmed gland genes. Gene ontology analysis identified twenty genes encoding enzymes, six transporters, three channels, two transcription factors, as well as thirty diverse other molecular functions, mainly receptors and enzyme regulators and eight genes, for which no molecular function could be assigned so far.

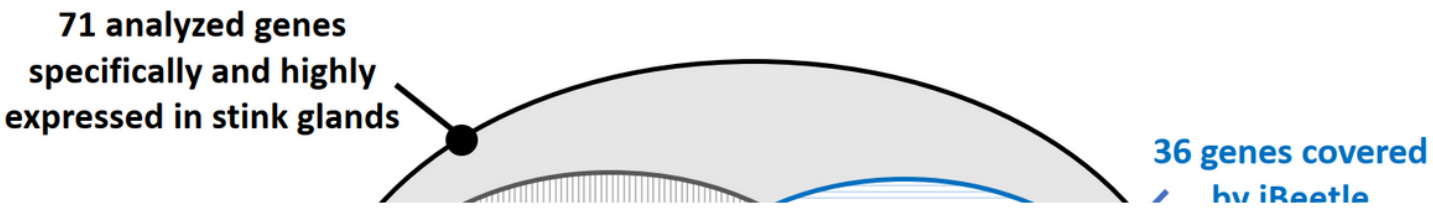


**Figure 3**

Comparison of transcriptome analysis and iBeetle phenotypic screen. Of 71 genes identified by a transcriptomics approach to be highly and specifically expressed in gland tissue, 29 genes showed a gland phenotype in knock-down situations [40]. Of the 71 genes, 36 genes were analyzed during the first and second phase of the iBeetle screen. 13 thereof had shown gland phenotypes in the transcriptomics-based analysis [40], but only was confirmed (conf.) in the iBeetle screen. Two genes were not analyzed (n.a.) for a stink gland phenotypes as they were only part of the pupal screen of the first phase) and seven (6+1) genes non detected (n.d.), of which at least one should have been detectable due a easily visible strong melanized gland content phenotype.

## Supplementary Files

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- Table1.png
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