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Inotocin, a potential modulator of reproductive behaviours in a biparental beetle, *Lethrus apterus*

Nikoletta A. Nagy [a,b,*], Zoltán Németh [a,b], Edit Juhász [b], Szilárd Póliska [c], Rita Rácz [a,b], Johanna Kiss [a,d], András Kosztolányi [e], Zoltán Barta [a,b]

[a] MTA-DE Behavioural Ecology Research Group, Department of Evolutionary Zoology, University of Debrecen, H-4032 Debrecen, Egyetem tér 1, Hungary
[b] Department of Evolutionary Zoology and Human Biology, University of Debrecen, Debrecen H-4032, Egyetem tér 1, Hungary
[c] Genomic Medicine and Bioinformatic Core Facility, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Debrecen H-4032, Egyetem tér 1, Hungary
[d] MTA-DE "Lendület" Evolutionary Phylogenomic Research Group, H-4032 Debrecen, Hungary
[e] Department of Ecology, University of Veterinary Medicine Budapest, Budapest, Hungary

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ABSTRACT

Several members of the highly conserved oxytocin/vasopressin neuropeptide family are involved in the regulation of reproductive and affiliative behaviours in numerous vertebrate and invertebrate species. Here we investigate gene expression patterns of inotocin, the insect ortholog of this peptide family, and its receptor to decipher their possible role in the control of reproductive behaviour in a beetle, *Lethrus apterus*, with biparental care. In an experiment performed on individuals of a wild population, we found that inotocin is not related to the control of water balance in this species because expression patterns did not change as a response to drought exposure. The expression levels of inotocin and its receptor, however, increased over the reproductive season i.e., when behaviour shifts from pair formation to parental care, suggesting that inotocin might be involved in the regulation of parental care in this insect. No difference was, however, found between sexes; a finding which might indicate that inotocin plays a similar role in both parents.

1. Introduction

Oxytocin, vasopressin and their orthologs compose a highly conserved neuropeptide family as indicated by their appearance in numerous animal taxa. Orthologs were found in mammals, birds, amphibians, fishes and also in invertebrates including insects, molluscs, and even earthworms and nematodes (Beets et al., 2013). These peptides are of great interest, as not only their sequences are homologous but their roles in physiology and behaviour appear to be remarkably similar even in distant species (Hanoune, 2010).

One of the ancient roles of this peptide family is related to the regulation of water balance (Hanoune, 2010). For example, vasotocin has antidiuretic functions in birds and amphibians (McCormick and Bradshaw, 2006), similarly to the role of vasopressin in mammals (Banerjee et al., 2017). An osmoregulatory role of vasotocin was also described in fish (Balment et al., 2006). Moreover, a relationship between vasopressin-like peptides and water homeostasis was also found in several invertebrate species, including leeches (*Whitmania pigra* and *Erpobdella octoculata*; Fujino et al., 1999, Salzet et al., 1993) and pleated sea squirt (*Styela plicata*; Ukena et al., 2008).

Another widespread role of these peptides is their involvement in the modulation of affiliative and reproductive behaviours across distant taxa as worms, molluscs, insects and vertebrates (Donaldson and Young, 2008). For instance, oxytocin is responsible for maternal nurturing, and vasopressin affects paternal care and mate guarding in the prairie vole (*Microtus ochrogaster*; McGraw et al., 2010). In addition, activation of oxytocin receptor is required for stable pair formation in the zebra finch (*Taeniopygia guttata*; Klatt and Goodson, 2013), and vasotocin induces egg-laying behaviours in the rough-skinned newt (*Taricha granulosa*; Moore et al., 1992). Among invertebrates, lysine-conopressin-G regulates male copulatory behaviour in the great pond snail (*Lymnaea stagnalis*; van Kesteren et al., 1995), annetocin plays a role in egg-laying behaviour in the brandling worm (*Eisenia fetida*; Oumi et al., 1996), and arginine-conopressin-G, annetocin, as well as hirudotocin activate a

* Corresponding author at: MTA-DE Behavioural Ecology Research Group, Department of Evolutionary Zoology, University of Debrecen, H-4032 Debrecen, Egyetem tér 1, Hungary.
E-mail address: nnolett@gmail.com (N.A. Nagy).

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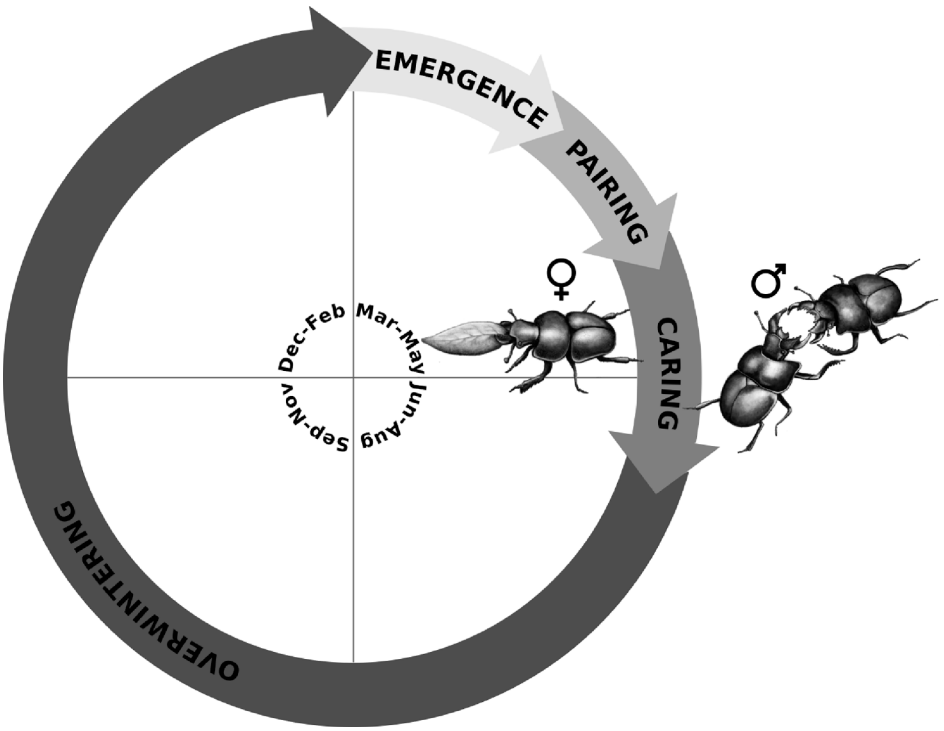

Fig. 1. The breeding cycle of *Lethrus apterus*. Beetles are only active from early March to the beginning of June in Hungary. During the period of parental care, females collect leaves for the offspring while males protect the nest from intruders.

sequence of reproduction-related behaviours in a medicinal leech (*Hirudo verbana*; Wagenaar et al., 2010).

The insect ortholog of the oxytocin/vasopressin peptide family, inotocin, was found in 22 out of 29 insect orders investigated using genome annotations (Liutkeviciute et al., 2016). Within the 20 previously examined coleopteran species, all but one have only one type of inotocin with the same amino acid sequence. The genome of *Leptinotarsa decemlineata*, however, codes two different types of this peptide, both of them differ in the 8th amino acid position from the inotocin of other coleopterans (Liutkeviciute et al., 2016).

Even though inotocin appears to be widespread across insects, its function and the specific behaviours it affects have only been investigated in a few species (reviewed in Muratspahić et al., 2020). In migratory locust (*Locusta migratoria*) and red flour beetle (*Tribolium castaneum*), inotocin was found to be involved in controlling water balance (Proux et al., 1987, Aikins et al., 2008). In addition, an association between inotocin and metabolism was discovered recently in ants (Liutkevičiūte et al., 2018). A potential function of inotocin in reproductive or social behaviour has also been suggested in black garden ant queens (*Lasius niger*, Chérasse and Aron, 2017). However, in this study, only the expression of inotocin receptor, but not the hormone was measured. Based on the examples above and the conserved functions of the oxytocin/vasopressin system over evolutionary time, one would expect that besides the ancient diuretic function, inotocin also modulates the expression of social behaviour, however, evidence is scarce. Thus, it is an intriguing question whether inotocin may have a function in shaping the social behaviour of insects similar to that of the vertebrate orthologs.

In this paper, we report a new form of inotocin for beetles and its receptor from *Lethrus apterus,* a beetle species that has well-developed biparental care (Wilson, 1971). *Lethrus apterus* is only active during the reproductive period, which usually lasts from March to June (Fig. 1). After finding a mate, the pair prepares the underground nest together (Rosa et al., 2017). Once the nest is excavated, a division of labour can be observed between sexes: the female collects and stores up leaves in the brood chambers for each offspring while the male guards the nest from intruders (Kosztolányi et al., 2015, Rosa et al., 2018, Kiss et al., 2020a).

Based on the knowledge collected so far on its function (see above), inotocin in *Lethrus apterus* may regulate water balance and/or play a role in the control of reproductive social behaviours, probably including parental care. To uncover the role of inotocin in water management we experimentally exposed beetles from a wild population to different levels of humidity and examined the gene expression of both the pre-prohormone (hereafter In) and the receptor (hereafter InR). If inotocin has a function in controlling reproductive behaviour in general and parental care in particular, one would expect that the expression levels of In and InR increase over the breeding season in concurrence with the change in the behaviour of beetles shifting from mate searching, through pair formation to parental care. To investigate this possibility, we sampled beetles over the reproductive season in the field to measure the gene expression level of In and InR. Coinciding with this sampling we also performed field surveys to observe the reproductive behaviours of the beetles.

2. Materials and methods

2.1. Effect of humidity on inotocin and receptor levels

An experiment was performed on 24th April 2018 at Susa, Hungary (48°16′27″N, 20°15′08″E) to examine the effect of humidity on the expression of In and InR. For this purpose, individuals were collected in the field during their daily activity, and three experimental groups were created (dried, saturated and control), each consisting of five males and five females. Individuals in the dried and saturated groups were placed for four hours in 50 ml conical centrifuge tubes which contained 20 ml indicating silica gel (Qingdao Fraken International Trading Co. Ltd, Orange indicating silica gel) which is a widely used hygroscopic agent in insect desiccation experiments (Andersen et al. 2010, Pallarés et al. 2016). For the dried group, water was completely eliminated from the silica gel by heating at 180 °C in a dry heat sterilizer for two hours. In these tubes 12.7 ± 1.34% (mean ± SD) humidity was measured with a

Table 1
Primers used to measure the expression levels of the two targets and the two reference genes by RT-qPCR. Acc. no: GenBank accession number; A.L.: amplicon length; M.T.: melting temperature; E: efficiency; R^2: regression coefficient.

Gene	Acc. no	Primer sequence (5′-3′)	A.L. (bp)	M.T. (°C)	E (%)	R^2
In	MT920922	F: ATGTTTAAAATCGTCGTTC R: CAAGCAACCGAAAGTTCCA	169	81.0	109.37	0.90
InR	MT920923	F: AGATGTCCATGAACAATACG R: CGTTATATTCCTGGTGACC	331	80.08	101.44	0.97
Ribosomal protein L7A	KY786277	F: TAGCGACTCAACTGTTCAAGG R: CCTCAATTGGATCGACGTCATGTG	224	84.8	99.54	0.95
Ribosomal protein L18	KY786276	F: TTGTAACCACATGAACGCCTACG R: AGTTAGCTTTACGTTCACCTACTGG	186	85.2	99.75	0.96

digital hygrometer (Exo Terra PT2477 Digital Hygrometer). For the saturated group, silica gel was saturated in an experimental box in 95% humidity for six hours. In tubes filled with saturated silica gel, the humidity was 41.8 ± 3.85% (mean ± SD). Sponge plugs were used as barriers to separate beetles from silica gel during the experiment. For the control group, samples were taken immediately after field collection, whereas sampling of individuals from experimental groups was performed after the four hours of treatment. Based on a study on the Colorado potato beetle (*Leptinotarsa decemlineata*), gene expression changes can be detected after three hours of low humidity exposure (Zhang et al. 2008). During sample collection for the control group, relative humidity was 53.75 ± 8.02% in the field based on four measurements.

Because the brain of *L. apterus* is surrounded by other tissues from which it is hard to distinguish with the naked eye in the field, we decided, similarly to other studies of expression analysis in insects (Chérasse and Aron, 2017; Stafflinger et al., 2008), to remove all tissues from the head capsule. Each sample was immediately put in separate 1.5 ml tubes filled with 600 µl RNAlater® Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA) in order to inhibit RNase enzyme activity. Tissue samples were collected in the field in less than five minutes after collecting individuals. Samples were stored at −20 °C in the laboratory until RNA extraction. Sample collection was approved by the Northern Hungarian Inspectorate for Environment Protection and Nature Conservation (No. 9007–8/2014) since *Lethrus apterus* is a protected species in Hungary.

2.2. Behavioural observation of Lethrus apterus

To investigate the phenology and reproductive behaviour of *L. apterus*, line transect monitoring was conducted between 16 March and 23 May in 2016 in the Susa population. During the survey, we walked on four previously selected line transects (length 285.45 ± 7.08 m, mean ± SD) covering an area of approximately 1 ha. The activity of every detected specimen was recorded in four categories (resting, travelling, leaf-carrying, fighting). In the case of leaf-carrying, the taxonomic identity of the carried leaf was also determined. Surveying was usually carried out between 10 am and 6 pm depending on weather conditions and in total we had 40 survey days.

2.3. Sample collection for measuring inotocin and receptor expression

Sample collection over the reproductive period was carried out in northern Hungary over a two year period. In 2015, samples were collected at Dorogháza (47°59′29″N, 19°53′36″E) on three occasions representing the beginning, middle and end of the period of parental care (16th April, 4th May and 28th May). In 2016, samples were collected at Susa on five occasions to cover the entire active season from emergence to end of parental care (18th March, 1st and 15th April, and 2nd and 17th May). On each sampling date, head samples were taken from eight males and eight females, except on 18th March 2016 when we sampled only four male and four female beetles because of the scarcity of individuals - the breeding season had just been started at that time.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The isolated RNA was eluted in 15–30 µl RNase-free water, depending on the pellet size. Concentration and quality of the extracts were determined by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was eliminated using RQ1 RNase-Free DNase (Promega, Madison, VI, USA) just before the reverse transcription. First-strand cDNA synthesis was performed from 1 µg DNA-free RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

2.5. Gene identification and primer design

Genes for inotocin and its receptor were identified in the draft genome of *L. apterus* (GenBank accession number: GCA_018397195.1) based on homologous sequences from *Tribolium castaneum* (GenBank accession numbers: NP_001078831 and NP_001078830 for In and InR, respectively) using BLASTX (BLAST 2.2.31+; Camacho et al., 2009). Gene structures were determined using transcriptome data (unpublished data). For the two genes (GenBank accession numbers in Table 1), primers were designed manually using the web-based Sequence Manipulation Suite (Stothard, 2000). In addition, Multiple Primer Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) was applied to check that our primers do not form any secondary structures. For normalization, two reference genes were used, ribosomal protein L7A and L18, for which primers described in Nagy et al. (2017) were applied. Sequences of the primers are shown in Table 1.

2.6. Real-time quantitative PCR

RT-qPCR was performed on a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) from the six times diluted cDNA using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and ROX Passive Reference Dye (Affymetrix, Santa Clara, CA, USA). Amplifications were carried out under the following conditions: initial denaturation at 95 °C for 10 min followed by 40 cycles of 10 sec at 95 °C and for 1 min at the optimal annealing temperature. For melting curve analysis, the temperature was raised from 65 °C to 95 °C in sequential steps of 0.05 °C for 1 sec. Three technical replicates were performed for each biological sample, and the average cycle threshold (Ct) values of triplicates were calculated. A no-template control was included in each run for each gene to exclude primer-dimers and non-specific contamination. Standard curves were created with five 5-fold dilution series from cDNA samples. Products of the qPCR were sequenced in order to check whether the correct sequences were amplified. Due to logistical reasons, In expression was measured only in samples from Susa from 2016 and in samples from the drying experiment (Susa, 2018), whereas InR was measured in samples from Dorogháza (2015), Susa (2016) and the drying experiment (2018). Prior to statistical analysis, expression of In and InR were normalized with the geometric mean of the two reference genes. As samples were collected

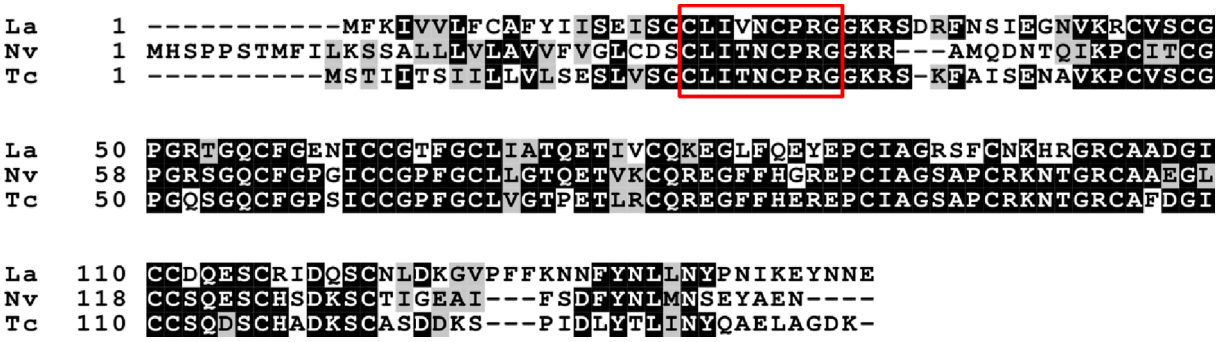

Fig. 2. Multiple sequence alignment of the inotocin preprohormone (In) of the *Lethrus apterus* (La, GenBank accession number: MT920922), the biparental *Nicrophorus vespilloides* (Nv, XP_017777933) and the model species *Tribolium castaneum* (Tc, NP_001078831). The red box highlights the mature peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

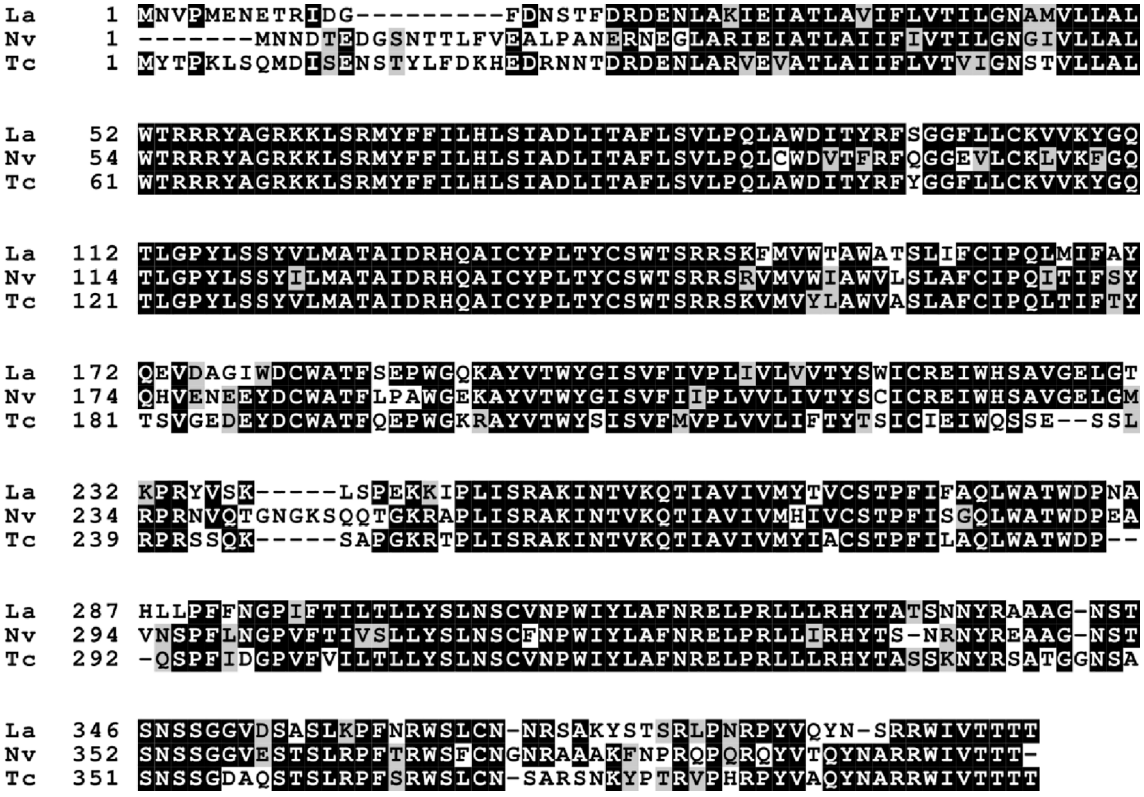

Fig. 3. Multiple sequence alignment of the inotocin receptor (InR) of *Lethrus apterus* (La, MT920923), *Nicrophorus vespilloides* (Nv, XP_017769191) and *Tribolium castaneum* (Tc, NP_001078830).

from natural populations, no control group was determined, thus for the $2^{-\Delta\Delta Ct}$ method, the sample with the highest Ct value was used as the control sample for each gene (Pabinger et al., 2014).

2.7. Statistical analysis

All statistical analyses were carried out using the R statistical environment version 3.6.0 (R Core Team, 2019). Before analyses, the expression values were log-transformed. Significance of date or treatment and sex and their interactions were investigated by linear models in each site separately. During model selection, non-significant interactions were excluded (all $p>=0.05$). All main effects of interest were retained in the final models and their significance were investigated with F-test. Pairwise post-hoc comparisons of the expression levels of In and InR between sampling dates were performed using the "emmeans" package and Tukey adjustment (Lenth, 2020).

In the case of In measured in both the drying experiment and the seasonal pattern analysis, expression levels split into two groups (Fig. S1). To search for the reason behind the bimodal distribution, possible confounding effects were investigated (see Appendix for details). However, none of these effects explained the apparent clustering of expression levels. Therefore, a two-level factor explaining the expression groups was included in the linear models to maintain the unimodal normal distribution of errors assumption of the models. The cutoff values for the two groups were determined as the minimum of the estimated density function of expression values by two-group mixture models using the "mixsmsn" package (Prates et al., 2013).

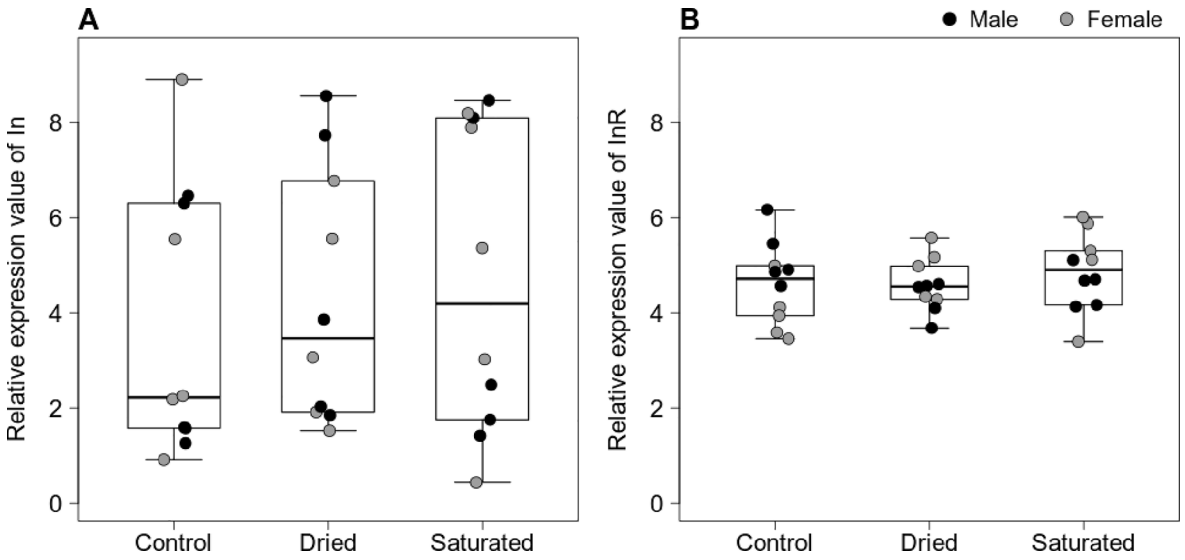

Fig. 4. Effect of drying experiment on the expression patterns of inotocin (In) and inotocin receptor (InR). Expression of In (A) and InR (B) of both sexes in the different treatment groups (n = 30). Within each box, horizontal black lines denote median values; boxes extend from the 25th to the 75th percentile of each group's distribution of values; vertical extending lines denote the minimum and maximum values. Original data points are represented as dots shaded black for males and grey for females.

Table 2
Results of linear model selections on the gene expression levels of inotocin (In) and its receptor (InR) from different years. Effect sizes of the final models (see Materials and methods) are presented as adjusted r-squared. Significant results are highlighted in bold.

Year	Gene	Effect	dfs	F value	p value
2015	InR ($r^2 = 0.072$)	Date	2,30	1.921	0.164
		Sex	1,30	1.822	0.187
2016	In ($r^2 = 0.897$)	Date	4,43	10.645	**<0.001**
		Sex	1,43	0.627	0.433
	InR ($r^2 = 0.25$)	Date	4,44	5.335	**0.001**
		Sex	1,44	0.156	0.695
2018	In ($r^2 = 0.876$)	Drying experiment	2,25	0.998	0.383
		Sex	1,25	3.227	0.085
	InR ($r^2 = 0.039$)	Drying experiment	2,26	0.418	0.663
		Sex	1,26	3.328	0.079

3. Results

3.1. Structure of inotocin and its receptor

Both In and InR were identified in the draft genome of *Lethrus apterus*. Based on the comparison of mRNA sequence and the genome, no intron intermits the gene coding for the 150 amino acids long In from which the mature nine amino acids long peptide is formed by cleavage steps. Compared with the mature inotocin sequences of beetle species reported in Liutkeviciute et al. (2016), we found that valine instead of threonine is coded in position 4 of the nonapeptide of *L. apterus* (Fig. 2). However, in the neurophysin part, the highly conserved 14 cysteine residues were identified similarly to other species (Stoop, 2012).

In the case of InR, the comparison of mRNA and genomic sequence revealed 5 introns in the gene. The whole protein consists of 397 amino acids and has seven transmembrane domains that are characteristic of G protein-coupled receptors (Fig. 3).

3.2. Manipulation of humidity

No significant difference was found among the experimental groups either in the expression of In (Fig. 4A, Table 2) or InR (Fig. 4B, Table 2). Expression of In was marginally higher in females (ß=-0.672 ± 0.37, Table 2) whereas InR expression was marginally higher in males (ß=0.478 ± 0.26, Table 2).

3.3. Seasonal variation in behaviour of Lethrus apterus

At the beginning of the active season, males were observed more frequently, by contrast, in the second half of the season females tended to be more active (Fig. S8A, S8B). An increased number of leaf carrying events was observed in the second half of the breeding period of which the majority was done by females (Fig. S8C, S8D). A large proportion of the collected leaves were classified as members of the Fabaceae family, especially later in the breeding season (Fig. 5A).

3.4. Seasonal expression of inotocin

Date had a significant effect on In expression (Table 2). Specifically, expression levels at the first two sampling dates differed significantly (or marginally) from the gene expression levels at the last three sampling dates which change was in line with the increase in the leaf collecting events (Fig. 5B, Table 3). On the other hand, no difference between sexes was detected (Table 2).

3.5. Seasonal expression of inotocin receptor

In the Dorogháza population (2015), expression of InR showed an increase with date but this was not significant (Fig. 6, Table 2, 3). In the Susa population (2016), on the other hand, InR expression levels changed significantly over the season (Table 2). Based on the post-hoc comparisons, expression levels on the third date was only marginally higher than the first two sampling date, however, a peak of InR expression on the fourth sampling date was significantly higher than the expression on the first two sampling dates (Fig. 5C, Table 3). After this peak, InR expression tended to decrease at the end of the season. These seasonal changes in InR expression were consistent with the changes in the leaf collecting behaviour, i.e., the peak of InR expression coincided with the peak of carrying legume leaves (Fig. 5A). Sex had no significant effect in either population (Table 2).

4. Discussion

Our findings support our expectation that inotocin may be involved

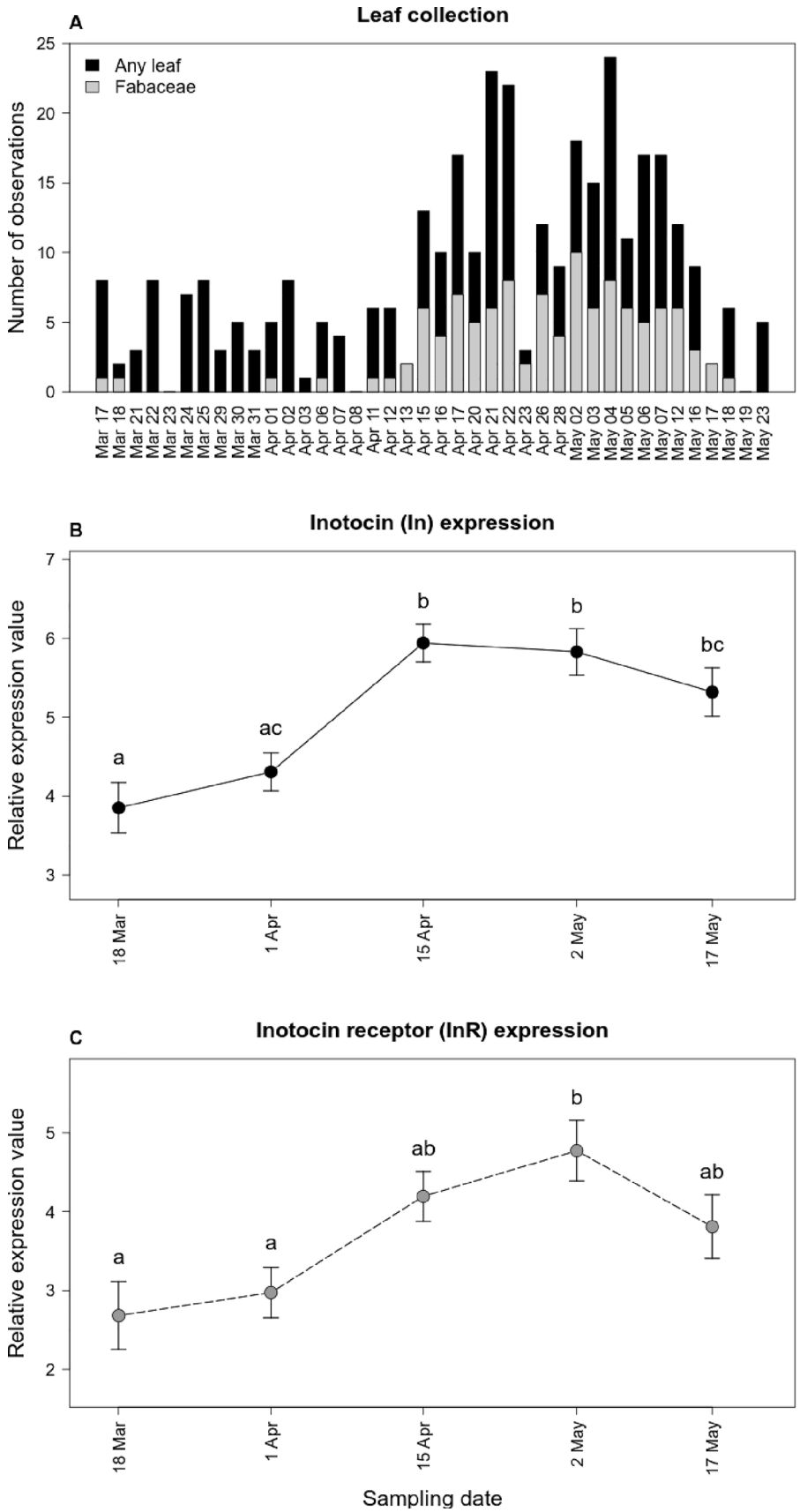

Fig. 5. Number of leaf collection events (A) and gene expression changes (B, C) of both sexes during the active period of *Lethrus apterus* in 2016, Susa. (A) Grey bars represent the collection of a leaf of a legume species, whereas black bars show leaves of any other taxa. Expression patterns of (B) inotocin (In) and (C) inotocin receptor (InR) represented as estimated marginal mean ± SE (n = 80); dates with different letters differ significantly (Tukey-adjusted comparisons).

Table 3
Pairwise post-hoc comparisons of inotocin (In) and inotocin receptor (InR) gene expression levels among dates in the Dorogháza and the Susa populations. Adjusted P values were calculated with the Tukey method. Results were averaged over sex, and, in the case of In, over the two-level factor that explains the expression groups (see Materials and methods). Significant comparisons are in bold.

Contrast		Estimate ± SE	p value
2015, Doroghaza			
InR (df = 30)			
16 Apr	4 May	−0.817 ± 0.599	0.3720
16 Apr	28 May	−1.088 ± 0.582	0.1654
4 May	28 May	−0.271 ± 0.622	0.9007
2016, Susa			
In (df = 43)			
18 Mar	1 Apr	−0.455 ± 0.399	0.7842
18 Mar	15 Apr	−2.089 ± 0.402	**0.0001**
18 Mar	2 May	−1.976 ± 0.439	**0.0005**
18 Mar	17 May	−1.466 ± 0.441	**0.0150**
1 Apr	15 Apr	−1.633 ± 0.346	**0.0002**
1 Apr	2 May	−1.521 ± 0.386	**0.0026**
1 Apr	17 May	−1.010 ± 0.379	0.0764
15 Apr	2 May	0.112 ± 0.368	0.9980
15 Apr	17 May	0.623 ± 0.397	0.5239
2 May	17 May	0.511 ± 0.433	0.7627
InR (df = 44)			
18 Mar	1 Apr	−0.289 ± 0.536	0.9827
18 Mar	15 Apr	−1.509 ± 0.534	0.0521
18 Mar	2 May	−2.088 ± 0.578	**0.0066**
18 Mar	17 May	−1.124 ± 0.591	0.3320
1 Apr	15 Apr	−1.220 ± 0.446	0.0650
1 Apr	2 May	−1.799 ± 0.493	**0.0059**
1 Apr	17 May	−0.835 ± 0.510	0.4831
15 Apr	2 May	−0.579 ± 0.494	0.7672
15 Apr	17 May	0.385 ± 0.511	0.9427
2 May	17 May	0.964 ± 0.552	0.4176

in the control of reproductive social behaviour in a beetle species, the *Lethrus apterus*. Gene expression of InR, as well as In, was found to change over the course of the breeding period, i.e., expression was higher later in the season when parental behaviours dominate the activities of both sexes (see also Fig. S8). In particular, changes in expression of In and InR were in accordance with the changes in legume leaf collection events. Legumes have high nitrogen content which may be necessary for fast larval development (Ohmart et al., 1985; Heisswolf et al., 2005). Examples of a similar expression pattern of oxytocin/vasopressin related peptides correlating with different forms of social behaviours can be found in vertebrates. In the biparental prairie vole, for instance, the expression of vasopressin gene was found to be higher postpartum in both males and females suggesting a role in parental behaviour (Wang et al., 2000). Furthermore, increased isotocin expression was related to affiliative behaviours toward the pair in a monogamous cichlid species, *Neolamprologus pulcher* (O'Connor et al., 2016). In the case of zebra finches, with the pair formation, the expression of both mesotocin and vasotocin increased in males as well as in females (Lowrey and Tomaszycki, 2014). Considering the conserved functions of the oxytocin/vasopressin peptide family, higher inotocin expression after pair formation can suggest a role in affiliative or parental behaviour in *Lethrus apterus*. Nevertheless, we cannot rule out alternative explanations for the changed expression pattern. A possible reason for this change may be the effect of the altered spectrum of collected plant species. The followings, however, make this alternative explanation less likely. First, leaves collected during the second part of the breeding season are mainly stored up as food for the larvae (Emich, 1884), hence the changed preference for plant species does not necessarily mean that the diet of the adults also changes during the breeding period. Second, legume leaf collection, which is driving the change in species composition, is largely carried out by females (Fig. S7), but gene expression patterns changed with the season in both sexes. We also have to note, however, that a study found an association between oxytocin/vasopressin related peptides and metabolism in an ant (Liutkevičiūte et al., 2018). Therefore, it is still possible that changing activity over the season influences gene expression pattern. Nevertheless, the activity pattern changes differently in sexes (Fig. S8) but no sex differences were found in gene expression patterns. One could also argue that the observed expression patterns might be related to changes in immunity during the breeding period. However, there is no obvious link between immune response and season (Kiss et al., 2020b), therefore, we would not expect that seasonal changes in In or InR gene expression correlate with changes in immunity. Other explanations may include seasonality of temperature or circadian rhythm. Albeit we cannot exclude the effect

Fig. 6. Expression pattern of inotocin receptor (InR) (estimated marginal mean ± SE) of both sexes (n = 48) during the reproductive period of *Lethrus apterus* in 2015, Doroghaza.

of changing weather conditions, e.g. the seasonally rising temperature in the natural environment, a possible relationship between the circadian rhythm and the gene expression changes seems unlikely since individuals were sampled at the same part of the day on each date in 2016 (Fig. S3A).

In the beetle *Tribolium castaneum* higher expression levels of In and InR were found in the head compared to other body parts by Stafflinger et al. (2008). Based on their results, Stafflinger et al. rejected the hypothesis that inotocin contributes to water balance regulation. By contrast, inotocin was found to exert an indirect effect on diuresis in the same species (Aikins et al., 2008). Furthermore, inotocin receptor has a role in desiccation resistance in an ant species, *Camponotus fellah* (Koto et al., 2019). We found that the expression of In and InR did not change as a response to desiccation stress. Although we did not measure the gene expression in other body tissues such as Malpighian tubules, which could also play a role in the regulation of desiccation resistance, our results are important as they suggest that the seasonal changes detected in the expression of both molecules were not influenced by the seasonally alternating humidity.

An interesting result of our study is that we identified a threonine-valine substitution in the fourth position of the inotocin of *L. apterus*. Threonine is the most common amino acid in the fourth position of the mature nonapeptide in arthropods, and also threonine was found in the fourth position in all other 20 beetle species studied to date (Liutkeviciute et al., 2016). This threonine-valine substitution was only identified in a highly social ant species earlier (Gruber and Muttenthaler, 2012).

Consistent bimodal distribution was found in the expression of In in two years which was explained neither by the sex nor any of the potential confounding effects that may have arisen during the sampling or laboratory procedures (see Appendix for details). Therefore, we suggest that the source of this clustering in the expression levels may be due to some unknown biological reasons. One explanation could be that individuals of a stable pair express In in higher or lower amount compared to unpaired individuals. Another possible cause can be age-related differences in reproductive history, as both first breeders and older breeders occur in the populations. For instance, in *Camponotus fellah* ant workers the expression of both In and InR increased with age (Koto et al., 2019). Unfortunately, information on either the reproductive state (i.e., paired or unpaired) or the age of the sampled individuals was not collected in this study. Therefore, investigating the background of this bias in In gene expression in beetles observed prior to sampling could reveal intriguing biological details at both the behavioural and molecular levels.

Even though numerous studies focusing on the oxytocin/vasopressin peptide family have been published, few have investigated the time course of expression, especially among invertebrates. Only two studies have been published to date in which receptor levels were measured over a period of time. Levoye et al. (2005) examined vasopressin-related receptor expression in a leech species, *Theromyzon tessulatum* during its reproductive period and found that the expression increased in adult individuals during the reproductive maturation until egg-laying. Chérasse and Aron (2017) measured InR expression over the course of the lives of black garden ant queens till colony foundation. Their results showed that expression of InR was lower during colony foundation than before mating which suggests a relationship between reproductive state and inotocin. Exploring and comparing the changes in expression of oxytocin/vasopressin related peptides and their receptors in a wide range of species with different types of reproductive strategy during their breeding period could reveal evolutionary processes modulating the functions of these molecules (Hofmann et al., 2014).

5. Conclusions

Based on our results we provide a new perspective on the potential functions of inotocin, the insect oxytocin-like hormone, i.e., it might be involved in the regulation of the social behaviours of a biparental beetle. However, it is important to note that from expression data one can only infer the activity of the gene, but not the actual levels of hormones in the haemolymph. Therefore, examining inotocin at the peptide level could further elucidate about its function in the control of parental behaviours. Further comparative investigations into the regulatory roles oxytocin-like neuropeptides play in invertebrate species are important to understand the evolution of biparental care and its neuroendocrine regulation.

Author contributions

N.A.N., Z.N., A.K. and Z.B. designed and directed the project. N.A.N., E.J., R.R., Z.N. and Z.B. collected the samples. N.A.N., R.R. and S.P. carried out the laboratory work. N.A.N., A.K. and Z.B. performed the statistical analyses. J.K. and R.R. accomplished the behavioural sampling. N.A.N. took the lead in writing the manuscript and all authors provided critical feedback to it.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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